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Aus der klinisch-physiologischen Abteilung, Serafimerlasarettet, und der pharmakologischen Abteilung des Karolinska Institutes, Stockholm, Schweden.

Untersuchungen über Eigenschaften und Verwendungsmöglichkeiten eines flexiblen Extremitätenplethysmografen.

Von

KLAUS GRAF und ALLAN WESTERSTEN.

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Abstract.

GRAF K. and A. WESTERSTEN. Untersuchungen über Eigenschaften und Verwendungsmöglichkeiten eines flexiblen Extremitätenplethysmografen. *Acta physiol. scand.* 1959. *46*. 1—18. — The flexible extremity plethysmographs described by DOHN were investigated on a dummy and in situ and found especially suitable for blood flow measurements by means of occlusion plethysmography. The models for forearm and calf could be used also on moving subjects, *e. g.* performing physical work. The plethysmographs consist of thin air-filled rubber cuffs. Volume changes of the extremity caused proportional changes of the filling pressure of the plethysmographs, which at rest was 40 mm H_2O and during venous occlusion increased by less than 10 mm H_2O. The pressure changes were continuously recorded on a capacitance manometer. Some properties and applications of the plethysmographs are described.

Quantitative Durchblutungsmessungen an menschlichen Extremitäten sind bis heute in verlässlicher Form nur mit der Venenverschlussplethysmografie möglich. Bei der arbeitenden Versuchsperson wird ihre Anwendung jedoch schwierig. Die bekannten

grossvolumigen Plethysmografen (LEWIS und GRANT 1925, GRANT und PEARSON 1938, BARCROFT und EDHOLM 1943, ABRAMSON 1944, KONDO *et al.* 1947, GREENFIELD 1954, HESS 1954, IMIG *et al.* 1955, weiteres bei BARCROFT und SWAN 1953) fixieren die Extremität in solchem Ausmass, dass Bewegungen des Armes oder Beines meist zu Artefakten führen, welche die Auswertung der Registrierung nicht mehr erlauben. Vergleichende Untersuchungen mit verschiedenen Plethysmografen zeigten uns, dass verwertbare Verschlussplethysmogramme an sich bewegenden Versuchspersonen sehr gut mit dem von DOHN (1956 a) beschriebenen flexiblen Extremitätenplethysmografen registriert werden konnten. Dieses Gerät ist bisher vorwiegend zur Volumenplethysmografie benutzt worden (DOHN 1953, BERTELSEN und DOHN 1953, JANSEN, BERTELSEN und DOHN 1956, DOHN 1956 b). Nennenswerte Erfahrungen über eine Anwendung auch bei verschlussplethysmografischen Durchblutungsmessungen liegen noch nicht vor. Vor Beginn weiterer Versuche schien daher eine Prüfung bzw. Nachprüfung der Verlässlichkeit, sowie der Verwendungsmöglichkeiten des Verfahrens, besonders im Hinblick auf verschlussplethysmografische Messungen, geboten. Die Ergebnisse dieser Untersuchungen werden zusammen mit einer modifizierten empfindlichen Druckmessanordnung zur elektrischen Registrierung der Plethysmogramme im folgenden beschrieben.

I. Prinzip des Plethysmografen.

Der Extremitätenplethysmograf besteht aus einer luftgefüllten, doppelwandigen Gummimanschette, deren innere Lamelle der Haut aufliegt. Der Plethysmograf umschliesst mit einem Füllungsdruck von 40 mm H_2O ein 5 cm breites Extremitätensegment. Volumenänderungen dieses Segmentes führen zu gleichgerichteten Druckänderungen im Plethysmografen. Volumenzunahmen des Segmentes von 1 %, die zur verschlussplethysmografischen Durchblutungsbestimmung ausreichen, erhöhen, abhängig von der Grösse der Plethysmografiemanschette, den Manschetteninnendruck um nur 2—7 mm H_2O, die Gesamtdruckbelastung des Gewebes beträgt also weniger als 50 mm H_2O (= 3.7 mm Hg). Weitere gemessene (mittlere) Zahlenwerte von verschiedenen Grössen der DOHN'schen Plethysmografiemanschetten s. Tab. I. Über ähnliche Hand- und Fussplethysmografen s. Kap. V.

Tabelle I.

Mittlere Zahlenwerte zu einigen Segmentplethysmografen.

Plethysmograf	Plethysmograf passend zu Zylindern mit:			Werte für das Gesamtsystem: Plethysmograf + Verbindungsschlauch + Druckkammer bei einem Füllungsdruck von 40 mm H_2O:			
Nr	Durchmesser (mm)	Volumen (ml)	Zirkumferenz (mm)	Luftinhalt (ml)	Drucksteigerung bei Vergrösserung des Luftinhaltes um 1 ml (mm H_2O)	Drucksteigerung (errechnet) bei Zunahme des Zylindervolumens um 1 % (mm H_2O)	Eigenfrequenz (Hz)
1	60— 65	140—165	190—205	75	4.4	6.0—7.0	25
2	70— 75	190—220	220—235	85	3.2	6.0—7.0	18
3	75— 80	220—250	235—250	165	1.4	3.0—3.5	21
4	85— 90	280—320	265—285	180	1.4	4.0—4.5	15
5	90— 95	320—350	285—300	335	0.8	2.5—2.8	14
6	95—105	350—430	300—330	500	0.6	2.1—2.6	15

II. Die Registrierung der Plethysmogramme.

Registriert werden die den Volumenänderungen der Extremität gleichgerichteten Druckänderungen im Plethysmografen. Da jene, Volumenpulsationen eingerechnet, in der Grössenordnung von 0.05—10.0 mm H_2O erfolgen, sind die heute zahlreich verfügbaren Geräte zur fortlaufenden elektrischen Druckregistrierung meist zu unempfindlich. Höhere Verstärkung ihrer Ausgangsspannung führt zu grösserer Inkonstanz der Anzeige. Andere Geräte, zur Messung geringer Druckdifferenzen eigens entwickelt oder modifiziert, sind im elektrischen Umformer- und Verstärkerteil gewöhnlich recht aufwendig (Kondo *et al.* 1947, Burch 1954, Imig, Sutfin und Hines 1955, Dohn, Gravenhorst und Jarløv 1956, Eckstein und Hamilton 1957, Winsor 1957, Scheppokat, Thron und Gauer 1958, Wood und Eckstein 1958). Wir benutzten einen einfacher aufgebauten Druckmessteil, mit dem noch Druckdifferenzen von 1 mm H_2O sehr empfindlich gemessen werden können.

1. *Die Apparatur zur Druckmessung.*

a) Der Druckrezeptor.

Er arbeitet nach dem Kondensatormikrofon-Prinzip (Näheres bei Tybjaerg-Hansen 1949) und ist in seinem Aufbau der von Dohn *et al.*

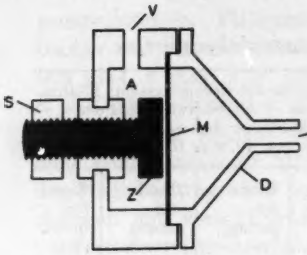

Abb. 1: Halbschematischer Querschnitt durch den Druckrezeptor. M (Druckmembran) und Z sind die beiden Platten eines Kondensators, deren Abstand durch den Feintrieb S beliebig variiert werden kann. D: Druckkammergehäuse. V und E: Eingänge zu den beiden Druckkammerabteilungen. Bei diesen Untersuchungen wurde der zu messende Druck über V und A auf M gegeben, während die gegenüberliegende Seite mit dem atmosphärischen Druck verbunden war.

(1956) beschriebenen Anordnung ähnlich. Die Membran M und der Zylinder Z bilden die beiden Platten eines Kondensators (Abb. 1). M besteht aus Phosphorbronze, hat einen Durchmesser von 15 mm und eine Dicke von 0.06 mm. Der Durchmesser von Z beträgt 10 mm. Das kapazitive System ist in der Druckkammer aus Messing D (Innenvolumen: 4 ml) untergebracht. Der Druck kann wahlweise über E oder V auf beide Seiten von M geleitet werden. Bei unseren Messungen wirkte er über V auf Raum A ein, so dass sich M bei Druckzunahme von Z entfernte. Der Raum auf der anderen Seite von M kann mit dem atmosphärischen Druck verbunden werden, wodurch Artefakte durch Luftdruckschwankungen oder Gerätevibrationen weitgehend gedämpft werden. Der Abstand von M und Z — und damit Kapazität und Empfindlichkeit der Apparatur — sind mit Hilfe des Feintriebes S beliebig zu variieren. Plattenabstände von 10—200 μ führen zu Kapazitäten von 65—18 pF.

b) Der Hochfrequenz-Messumformer.

Kapazitätsänderungen des Kondensators werden nach folgendem Prinzip, das gewisse Ähnlichkeiten mit einer von LILLY (1942) angegebenen Schaltung hat, in elektrische Potentialschwankungen umgewandelt (Abb. 2): In einem Oszillator mit ähnlicher Abstimmung der Anoden- und Gitterkreise variiert die negative Gitterspannung stark mit der Differenz in der Abstimmung. Dabei ergeben sich besonders grosse Potentialschwankungen bei Verwendung eines Gitterkreises mit hohem Q-Wert (= Güte des Kreises), z. B. einem Kristall. Die Potentialschwankungen werden über ein Filter, welches die überlagerte Oszillator-Wechselspannung aussiebt, dem nachgeschalteten Verstärkerteil zugeleitet. Im Bereich von 0—100 mm H_2O beträgt der Ausgang am Hochfrequenz-Messumformer (bei 30 pF): 180—60 mV/mm H_2O (Tab. II).

2. Eigenschaften der Druckmessapparatur.

Zur Registrierung diente ein Routine-EKG-Gerät als Verstärker und Schreiber (Mingograf 42, Hersteller: Elema, Stockholm). Mit diesem zeigte das Druckmessystem bei einer Kapazität von 30 pF (Plattenabstand: 0.06 mm) folgende Eigenschaften:

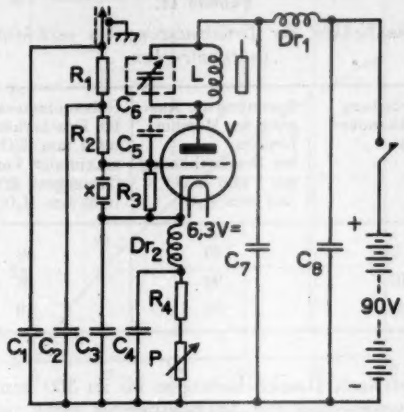

Abb. 2: Schaltschema des Hochfrequenzmessumformers.

V	= 6C4	C_1	= 0.015 μF
X	= Kristall 27 MHz	C_2	= 0.01 μF
Dr_1	= Dr_2 = Radiofrequenzdrossel 5 mH	C_3	= 0.01 μF
		C_4	= 0.01 μF
R_1	= 100 k Ω	C_5	= Anode-Gitterkapazität der Röhre V = 1.5 pF
R_2	= 200 k Ω		
R_3	= 100 k Ω	C_6	= Kondensator (Druckrezeptor) 30 pF
R_4	= 10 k Ω	C_7	= 0.01 μF
L	= Spule mit Eisenpulverkern	C_8	= 0.015 μF
P	= Potentiometer 10 k Ω		

a) Empfindlichkeit: Im Messbereich von 0—100 mm H_2O: 180—60 mV/mm H_2O (Tab. II). Diese Empfindlichkeit ist für verschlussplethysmografische Registrierungen (ausgenommen an der Fingerkuppe) optimal. Sie lässt sich vergrössern durch:

1. Verminderung des Kondensatorplattenabstandes mittels des Feintriebes S (in Abb. 1) oder

2. Ersetzen der Luft zwischen den Kondensatorplatten durch ein Medium mit höherer Dielektrizitätskonstante, etwa ein Silikonöl.

b) Konstanz der Anzeige: 2 Stunden nach Einschalten des gesamten elektrischen Systems betrug die Wanderung der Ausgangslinie bei Druckbelastung 0:

Amplitude in 1 Stunde: 3/56 mm H_2O.

Grösste Wanderungsgeschwindigkeit: 0.25/56 mm H_2O pro Minute. Maximaler Messfehler infolge Inkonstanz der Anzeige in 1 min: 0.5 % (Werte gemessen bei Raumtemperatur von 23 ± 0.5 ° C. Grössere Änderungen der Raumtemperatur führen zu grösserer Inkonstanz der Druckanzeige).

Tabelle II.

Empfindlichkeit der Druckmessung bei verschiedenen Anfangsdrucken.

Anfangsbelastung der Druckkammer (mm H_2O)	Spannung am Ausgang des Messumformers bei Druckerhöhung um 1 mm H_2O (mV/mm H_2O)	Galvanometerausschlag bei Druckerhöhung um 1 mm H_2O und maximaler Verstärkung (Mingograf 42) (mm/mm H_2O)
0	180	56
40	90	28
100	60	19

c) Hysteresis: Nach Druckbelastungen bis zu 300 mm H_2O war ein bleibendes Unterschreiten des Ausgangswertes nicht messbar.

d) Frequenzumfang: Eigenfrequenz des luftgefüllten Druckrezeptors (stationär bestimmt durch Anlegen sinusförmiger Druckschwankungen am Druckkammereingang mittels Tongenerator): 200 Hz.

e) Kurvenform der Druckanzeige: Von 0—100 mm H_2O Druckbelastung wird die Empfindlichkeit der Druckanzeige in dem in Abb. 3 gezeigten Masse kleiner. Der Grund ist die mit steigendem Druck und damit wachsendem Kondensatorplattenabstand abnehmende Kapazitätsänderung pro mm H_2O.

III. Eigenschaften des Plethysmografen.

1. Methodik der Modellversuche.

Die Eigenschaften des Plethysmografen wurden teilweise an einem Segmentphantom geprüft. Abb. 4 zeigt den Versuchsaufbau im halbschematischen Querschnitt. Ein hohler Messingzylinder von 75 mm Durchmesser enthielt an der Oberfläche eine 50 mm breite Vertiefung W von 3 mm. Auf dem Phantom sass die Plethysmografiemanschette (verwandt wurden die Grössen Nr. 2 u. 3 in Tab. I). Die innere Lamelle P_i erstreckte sich in ganzer Ausdehnung über W. P_a: äussere Lamelle des Plethysmografen. Füllung des Plethysmografen mit Luft sowie Druckmessung über D. Füllung von W mit Wasser über Konus E. T_1 und T_2: Kupfer-Konstantan-Thermoelemente (Referenzlötstellen in Dewar-Gefäss mit Wasser von Raumtemperatur, Registrierung der Thermoströme mit Threechannel recording thermometer, Hersteller: Pye, Cambridge). Durchströmung des Phantoms in C von A nach B mit Wasser aus einem Ultra-Thermostaten (Typ NB, Temperaturkonstanz: 0.05° C, Hersteller: Colora GmbH, Lorch). Rasche Temperaturänderungen in W waren leicht durch Änderungen der Thermostatentemperatur zu erreichen. Raumtemperatur bei allen Versuchen: 23 $\pm$ 0.5° C.

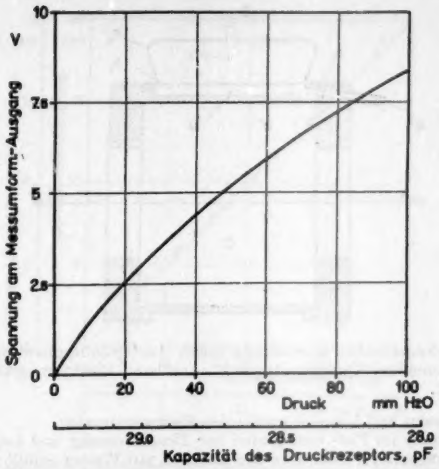

Abb. 3: Spannung am Ausgang des Druck-Messumformers in Abhängigkeit von der Druckbelastung und damit verbundenen Kapazitätsänderung des Druckrezeptors.

2. Beziehung zwischen wirklicher und plethysmografisch gemessener Volumenänderung.

Bei der üblichen Berechnung von Durchblutungswerten in ml/100 ml Gewebe · min kann ein Fehler dadurch auftreten, dass »the cuffs not undergoing the same changes in shape and volume by calibration as by the distension of the extremity« (DOHN 1956 a). DOHN fand in Modellversuchen eine Streuung des Quotienten aus wirklicher zu plethysmografisch gemessener Volumenänderung von ± 20 %. Wir prüften das Verhalten dieses Quotienten durch Vergleich der Drucksteigerungen (= Galvanometerausschläge) nach gleicher Vergrösserung des Luftvolumens im Plethysmografen und des Wasservolumens in W (Abb. 4). Die mit 2 verschiedenen Plethysmografen erhaltenen Ergebnisse zeigt Abb. 5. Volumenzunahmen des Zylindersegmentes bis zu etwa 10 %, dem 10-fachen Betrag also, der zur verschlussplethysmografischen Durchblutungsbestimmung ausreicht, ergaben in beiden Fällen gleich grosse Drucksteigerungen. Geringe Abweichungen liegen im unteren Teil der Kurve ausserhalb der Messgenauigkeit und werden erst bei grösseren Volumenänderungen deutlicher. Das Ergebnis dieser Messungen war auch nach wiederholter Montage derselben Plethysmografen auf dem Phantom das gleiche.

Die grösseren Schwankungen DOHNS sind dadurch erklärlich, dass er jeden Plethysmografen bei um 21 % wechselndem Phantomvolumen untersuchte (bei der sogenannten "kleinsten" und "grössten" Zirkumferenz, zu der die jeweilige Plethysmografiemanschette passte). Bei

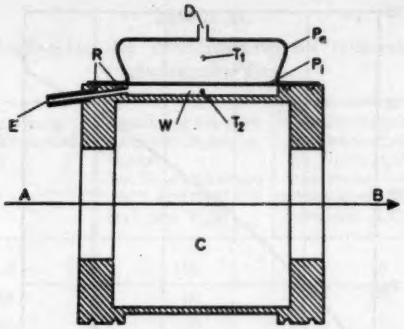

Abb. 4: Halbschematischer Querschnitt durch das Zylinderphantom, auf dessen obere Hälfte eine im Querschnittsprofil sichtbare Plethysmografiemanschette aufgespannt ist.

P_a und P_i: Äussere und innere Lamelle des Plethysmografen.
D: Öffnung im Plethysmografen zur Druckmessung und Luftfüllung.
W: Raum unter P_i, der über den Konus E mit Wasser gefüllt wird.
R: Rillen am erhöhten Rand des Phantoms zum eventuellen Festbinden der seitlich überstehenden Ränder des Plethysmografen.
T_1 und T_2: Kupfer-Konstantan-Thermoelemente (Drahtstärke: 0.08 mm).
C: Hohler, von A nach B mit Wasser aus einem Thermostaten durchströmter Innenraum des Phantoms.

der kleinsten Zirkumferenz fand auch DOHN (1958) im Bereich von Volumenänderungen, wie sie bei der Venenverschlussplethysmografie erfolgen, keine signifikanten Differenzen zwischen wirklichem und gemessenem Volumen, vielmehr traten die Abweichungen erst nach weiterer Dehnung der Plethysmografen auf. Werden daher nur ausreichend weite Plethysmografen verwandt, die spannungsfrei auf dem Extremitätensegment angebracht werden können, ist ein wesentlicher Kalibrationsfehler nicht zu erwarten.

Die Mitausdehnung der Ränder des Plethysmografen, die ebenfalls zu einer geringen Plethysmografenverformung und damit auch Änderung des Füllungsdrucks führen kann (DOHN 1956 a), darf bei den geringen Volumenänderungen bei verschlussplethysmografischen Messungen vernachlässigt werden. So ändert z. B. ein Zylindersegment von 250 ml Volumen (wie es durchschnittlich in die Messungen am Unterarm Erwachsener eingeht) seinen Durchmesser bei 1 % Volumenzunahme nur um 0.5 mm.

3. *Die Kurvenform der Druckanzeige im Gesamtsystem Plethysmograf + Druckrezeptor.*

Mit zunehmender Füllung nimmt der Elastizitätsmodul der Plethysmografiemanschetten und damit die Druckzunahme bei gleichen Vergrösserungen des Füllungsvolumens zu. Zur Kompensation diente die

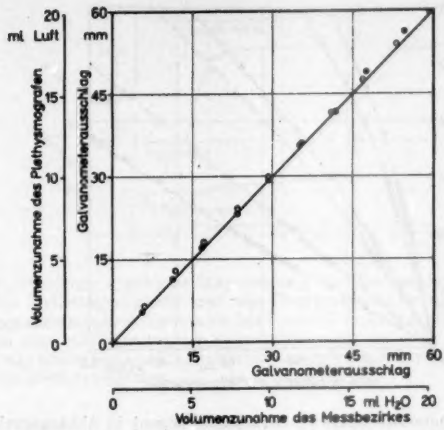

Abb. 5: Galvanometerausschläge (= Druckerhöhungen) bei Vergrösserung des Luftvolumens im Plethysmografen in Abhängigkeit der Galvanometerausschläge (= Druckerhöhungen) bei Vergrösserung des Zylindervolumens unter der inneren Plethysmografenlamelle.

● = Grösse Nr. 2,
○ = Grösse Nr. 3 des Plethysmografen in Tab. I.

Beginn der Messung bei einem Plethysmografenfüllungsdruck von 20 mm H_2O. 60 mm Galvanometerausschlag entsprechen einer Steigerung des Füllungsdruckes um 50 (von 20 auf 70) mm H_2O.

im Messbereich von 0—100 mm H_2O mit wachsender Druckbelastung abnehmende Empfindlichkeit der Druckanzeige (s. Kap. II. 2. e). Abb. 6 zeigt die sich für das Gesamtsystem Plethysmograf + Druckrezeptor ergebende Funktion aus Volumenzunahme und Galvanometerausschlag (= Druckzunahme).

Bei verschlussplethysmografischen Messungen betragen die Druckänderungen höchstens 10 (von 40 bis 50) mm H_2O. In diesem Bereich — in Abb. 6 durch verstärkt ausgezogene Teilstrecken angegeben — besteht eine für alle Plethysmografen weitgehend lineare Druck-Volumen-Beziehung.

4. Der Einfluss von Temperaturänderungen.

Die Innentemperatur der Plethysmografiemanschetten stellt sich auf ein von Haut- und Raumtemperatur abhängiges Gleichgewicht ein. Änderungen der Hauttemperatur können daher die Innentemperatur

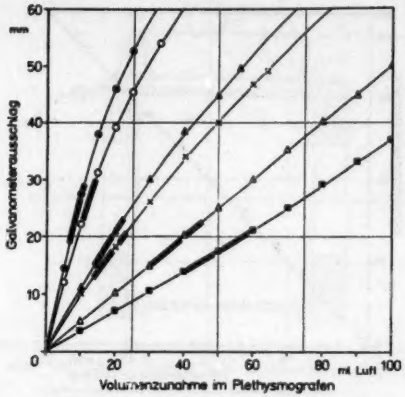

Abb. 6: Galvanometerausschläge (= Druckänderungen) in Abhängigkeit von der Luftvolumenzunahme in verschiedenen Plethysmografiemanschetten.

Nr. 1: ●; Nr. 4: ×;
Nr. 2: ○; Nr. 5: △;
Nr. 3: ▲; und Nr. 6: ■ der Tab. I.

Messungen von Füllungsdruck 20—100 mm H_2O. Die verstärkt ausgezogenen Teilstrecken entsprechen der Volumenzunahme bei Erhöhung des Füllungsdrucks um 10 (von 40 auf 50) mm H_2O und bezeichnen damit den Messbereich bei verschlussplethysmografischen Untersuchungen.

und damit den Füllungsdruck des Plethysmografen beeinflussen. Das Ausmass solcher Fehler wurde an dem Zylinderphantom (s. Kap. III, 1. u. Abb. 4) untersucht.

a) Die Grösse des Temperaturüberganges Haut—Plethysmograf.

Bei 23° C Raumtemperatur wird die Innentemperatur des Plethysmografen um etwa 25 % des Wertes beeinflusst, um den sich die Temperatur der Zylinderoberfläche unter der inneren Plethysmografenlamelle im Bereich von 30—40° C verändert (Beispiel s. Abb. 7). Steigerungen der Hauttemperatur um 1° C führen also zu einer Zunahme der Plethysmografen-Innentemperatur um 0.25° C.

b) Die Geschwindigkeit des Temperaturüberganges Haut—Plethysmograf.

Etwa 1 min nach einer steil einsetzenden Änderung der Zylinderoberflächentemperatur beginnt auch die Plethysmografeninnentemperatur mitzureagieren und erreicht erst einige Minuten später als jene ihren Endwert. Abb. 7 zeigt dies in einem Beispiel.

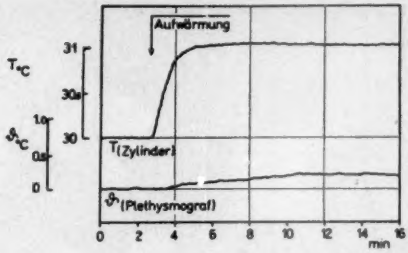

Abb. 7: Fortlaufende synchrone Registrierung der Temperatur T an der Oberschicht des Zylinderphantoms und der Temperaturänderung im Inneren der Plethysmografiemanschette (gemessen bei T_1 und T_2 in Abb. 4). Der Pfeil bezeichnet den Beginn einer Aufwärmung des Zylinderphantoms um etwa 1° C. Man beachte die erst 1 min später beginnende, weiterhin verzögerte und geringere Miterwärmung der Luft im Plethysmografen.

c) Die Temperatur-Druck-Beziehung im Plethysmografen.

Abb. 8 zeigt den Füllungsdruck der Plethysmografiemanschetten Nr. 2 und 3 (Tab. I) in Abhängigkeit von der Temperatur. (Druckmessung mit H_2O-Manometer nach Erreichen konstanter Werte.) Bei gleicher Temperatursteigerung wurde die Drucksteigerung mit zunehmender Plethysmografengrösse geringer. Sie betrug z. B. im Bereich von 30—40° C und 40—50 mm H_2O für Plethysmograf Nr. 2 annähernd 0.65 mm H_2O/° C, für Plethysmograf Nr. 3: 0.35 mm H_2O/° C.

Bei konstanter Umgebungstemperatur ist daher nicht zu erwarten, dass raschere Hauttemperaturänderungen, die selten mehr als 1° C betragen, eine nicht länger als 15 sec dauernde verschlussplethysmografische Messung verfälschen. Auch bei länger bestehenden Änderungen der Hauttemperatur werden die temperaturbedingten Änderungen des Füllungsdruckes 1 mm H_2O kaum überschreiten und sind daher auf die im Messbereich von 40—50 mm H_2O weitgehend lineare Volumeneichung (s. Kap. II. 3) ohne Einfluss.

5. Frequenzumfang.

Eigenfrequenzbestimmung der Plethysmografen Nr. 1—6 am Phantom und in situ bei einem Füllungsdruck von 40 mm H_2O. (Methodik: instationäre (durch einmalige Impulsgabe) und stationäre (mit Tongenerator) Erregung des schwingenden Systems. Näheres s. Frank 1903, Broemser 1930, Tybjaerg Hansen 1949 und Grosse-Brockhoff, Mürtz und Weiss 1956.) Die Werte lagen zwischen 14—25 Hz (Tab. I). Infolge unterschiedlicher Gummielastizität hatten mitunter grössere Plethysmografen höhere Eigenfrequenzen als kleinere: Volumenpulsationen im Bereich der Herzfrequenz können also formrichtig

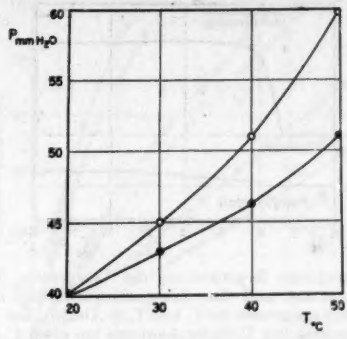

Abb. 8: Füllungsdruck der Plethysmografen Nr. 2 (○) und Nr. 3 (●) (Tab. I) in Abhängigkeit von der Plethysmografen-Innentemperatur.

registriert werden. Für eine amplitudengetreue Anzeige wären Eigenfrequenzen von wenigstens 50 Hz erforderlich (TYBJAERG HANSEN 1949).

IV. Verwendungsmöglichkeiten des flexiblen Plethysmografen.

Durchblutungsmessungen erfolgen mit diesem Gerät auf prinzipiell gleiche Weise wie mit den anderen bekannten Plethysmografen (Näheres s. BARCROFT und SWAN 1953). Abb. 9 zeigt die Versuchsanordnung bei der Messung am Unterarm. Die Plethysmografiemanschette sitzt über der proximalen, muskelreichsten Partie des Unterarmes, der in mindestens Herzhöhe gelagert wird. Oberhalb des Ellenbogens befindet sich die Okklusionsmanschette, distal vom Plethysmografen die Drosselungsmanschette. Letztere sitzt unmittelbar unterhalb des Plethysmografen. In Abb. 9 ist sie etwas tiefer angebracht, damit Lage und Form des Plethysmografen besser erkannt werden können. Bei jeder verschlussplethysmografischen Durchblutungsmessung wird zuerst die Drosselungsmanschette mit 200—300 mm Hg, etwas später (KERSLAKE 1949, GRAF 1959) dann die Okklusionsmanschette mit knapp unterdiastolischem Druck aufgeblasen. Nach der eigentlichen, 5—15 sec dauernden Durchblutungsmessung werden beide Manschetten wieder entlastet. Die Vorteile dieses Plethysmografen vor anderen (s. Einleitung) sind:

Abb. 9: Position des flexiblen Plethysmografen am Unterarm. Lagerung des Armes mit leicht aufwärts geneigtem Unterarm in Herzhöhe. An keiner Stelle liegt der Plethysmograf dem Tisch oder anderen Gegenständen auf. Die Drosselungsmanschette sitzt gewöhnlich unmittelbar distal vom Gerät, wurde hier jedoch zur besseren Demonstration von Form und Lage des Plethysmografen etwas weiter unterhalb angebracht.

1. Das Gerät ist derart fixiert (Abb. 9), dass es auch grösseren Bewegungen der Extremität mitfolgen kann, etwa bei Beinarbeit an einem Fahrradergometer, während der die Armdurchblutung der Versuchsperson gemessen werden soll (Abb. 10). Versuche, unter solchen Bedingungen mit einem der grossvolumigen, starren, die Extremität fixierenden Plethysmografen — ähnlich dem von BARCROFT und SWAN (1953) benutzten — verwertbare Ergebnisse zu erhalten, waren der starken Bewegungsartefakte wegen ohne Erfolg. Gleiche Erfahrungen mit einem solchen Plethysmografen machten RODDIE, SHEPHERD und WHELAN (1958), als sie Verschlussplethysmogramme während forzierter thorakaler Atmung registrierten. Unter diesen Bedingungen kann auch der DOHN'sche Plethysmograf nicht alle Bewegungsartefakte verhindern. Diese sind jedoch bei guter Lagerung der Versuchsperson so gering, dass sie die Auswertbarkeit der Volumenänderungen nach Venenverschluss nicht beeinträchtigen. Abb. 10 zeigt dies in einem Beispiel, in dem, nach unseren Erfahrungen aus mehr als 100 solcher Versuche, die Bewegungsartefakte noch recht gross sind.

Erst wenn die vom Plethysmografen umschlossene Muskulatur selbst Kontraktionen ausführt, machen die Artefakte eine Ausmessung des Volumenanstiegs unmöglich.

2. Zeitraubende Schwierigkeiten mit der Montage und Abdichtung des Plethysmografen bestehen nicht.

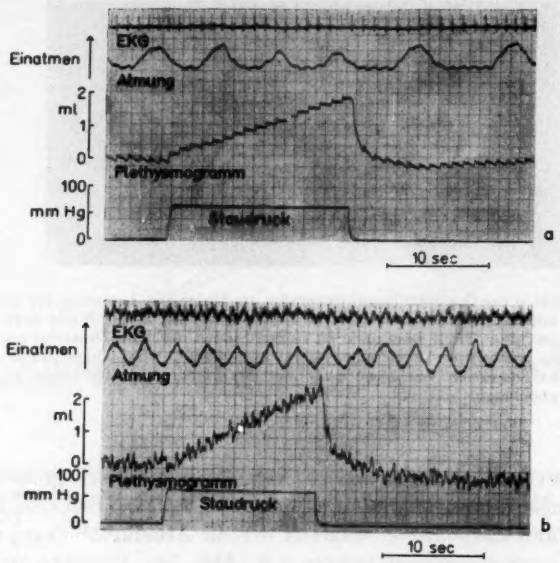

Abb. 10: Beispiele fortlaufender, direkter, elektrischer Registrierungen von Venenverschlussplethysmogrammen am rechten Unterarm mit synchroner Registrierung von EKG (Herzfrequenz), Atmung (Atemfrequenz) und Druck in der Okklusionsmanschette am rechten Oberarm. Originalkurven.

a) bei liegender, unbeeinflusster Versuchsperson (Durchblutung: 2.4 ml/100 ml Arm • min).

b) bei Beinarbeit im Liegen am Fahrradergometer, Belastung: 300 kpm/min (Durchblutung: 3.9 ml/100 ml Arm • min).

3. Der Plethysmograf umschliesst ein Extremitätensegment von genau 5 cm Höhe, dessen Volumen leicht mittels Wasserverdrängung, Zirkumferenzmessung oder, wohl am genauesten, Messung des Luftvolumens im Plethysmografen (DOHN 1956 a) bestimmt werden kann. Die quantitative Beziehung der gemessenen Stromzeitvolumenwerte zu einem definierten Gewebevolumen ist daher möglich. Der ideal einfache Straingauge-Plethysmograf Whitneys (1953) erfüllt diese wichtige Voraussetzung leider nicht, sondern ist auf eine rechnerische und empirische Bestimmung der in die Messung eingehenden Gewebsmenge angewiesen (CLARKE und HELLON 1957, CLARKE, GINSBURG und HELLON 1958).

Tabelle III.

Vergleichende Messungen der Handdurchblutung in ml/100 ml Hand · min bei 4 gesunden, liegenden, unbeeinflussten Versuchspersonen. Aufnahme der Werte mit derselben Registrieranordnung in 30 sec. Abstand abwechselnd an der rechten und linken Hand. Handplethysmograf nach FOLKOW, GRIMBY *und* THULESIUS (1958) *mit Wasserfüllung, Wassertemperatur: 33° C. Handplethysmograf modifiziert nach* FREEMAN (1935) *mit Luftfüllung, Luft von Raumtemperatur: 23° C.*

Versuchsperson	Flexibler Plethysmograf an rechter Hand	Plethysmograf nach FOLKOW *et al.* an linker Hand	Plethysmograf modifiziert nach FREEMAN an linker Hand
28 Jahre ♀	6.8	6.5	
	4.9	5.2	
	5.7	6.2	
	6.6	7.3	
	5.8	6.8	
	M = 5.95	M = 6.40	
26 Jahre ♂	9.8	8.2	
	8.7	7.9	
	6.4	9.9	
	10.1	7.7	
	8.4	8.5	
	M = 8.69	M = 8.44	
25 Jahre ♂	11.4		10.8
	9.3		6.9
	6.7		6.4
	7.5		7.0
	10.8		7.8
	M = 9.14		M = 7.78
23 Jahre ♂	6.9		5.7
	5.9		5.5
	6.6		6.2
	6.6		6.0
	6.2		6.0
	M = 6.44		M = 5.88

V. Flexible Hand- und Fussplethysmografen.

Flexible Plethysmografen wurden auch für die Messung an Hand oder Fuss konstruiert (DOHN 1956 a und 1958). Wir prüften Modelle, die aus einem Gummisack mit 2 Lamellen bestanden, aus denen ein Schlauch zur Druckmessung und Kalibrierung herausführte. Der Plethysmograf wurde über die Hand oder den Vorderfuss gezogen, wobei die innere Lamelle wieder der Haut

auflag. Anschliessend Füllung mit Luft bis zu einem Druck von 40 mm H_2O. Unter diesem Füllungsdruck war der Gummisack eben ganz entfaltet. Registrierung der Volumenänderung nach Venenokklusion an Hand- oder Fussgelenk über Druckänderungen und Kalibrierung wie beim Segmentplethysmografen (Kap. II). Bei längerdauernden Messungen kam es unter der inneren Lamelle mitunter zu beträchtlicher Feuchtigkeitsansammlung. Bewegungsartefakte waren jedoch auch hier geringer als bei starren Geräten, von denen wir verschiedene Modelle in Vergleichsversuchen prüften (Handplethysmograf modifiziert nach FREEMAN 1935, mit Luftfüllung, und Handplethysmograf nach FOLKOW, GRIMBY und THULESIUS 1958, mit Wasserfüllung). Tab. III zeigt, dass die mit den verschiedenen Plethysmografen an beiden Händen von Gesunden gemessenen Ruhedurchblutungswerte ähnlich gross waren, ein Befund, der auch bei synchroner Messung mit gleichen Plethysmografen erhoben wurde (BARCROFT und SWAN 1953).

Zusammenfassung.

1. Eigenschaften und Verwendungsmöglichkeiten von DOHN angegebener flexibler Extremitätenplethysmografen wurden, besonders hinsichtlich verschlussplethysmografischer Durchblutungsmessungen, am Modell und in situ untersucht.

2. Die Plethysmogramme wurden über eine besonders entwickelte Druckmessapparatur fortlaufend elektrisch registriert. Im Bereich von 0—100 mm H_2O betrug die Empfindlichkeit der Druckmessung 180—60 mV/mm H_2O. Höhere Empfindlichkeiten waren durch Änderungen am Druckrezeptor leicht zu erreichen. Die Eigenschaften der Druckmessapparatur wurden geprüft.

3. Die Gesamtdruckbelastung des Gewebes war kleiner als 50 mm H_2O. Bei verschlussplethysmografischen Durchblutungsbestimmungen betrug die Drucksteigerung im Plethysmografen weniger als 10 mm H_2O.

4. Physiologische Schwankungen der Hauttemperatur waren auf Kalibration und Kurvenform des Druckanstieges nach Venenverschluss ohne messbaren Einfluss.

5. Im verschlussplethysmografisch benutzten Messbereich war das Verhältnis zwischen wirklicher und gemessener Volumenänderung des Gewebes gleich 1.

6. Die Eigenfrequenz des gesamten Messystems lag bei Ver-

wendung verschiedener Plethysmografengrössen zwischen 14 und 25 Hz.

7. Die Segmentplethysmografen für Unterarm und Wade eignen sich besonders für Messungen, bei denen eine ruhige Lagerung der Versuchsperson nicht möglich ist, z. B. Arbeitsversuche, da sie auch grösseren Bewegungen der Extremität ohne Verschiebungen auf dem Gewebe mitfolgen. Unter gleichen Bedingungen erleichtern ähnliche, sackförmig ausgebildete Plethysmografen die Messung der Hand- und Fussdurchblutung.

Die Autoren danken den Herren Dr. K. Dohn für anregende Diskussionen und Kritik, Dr. B. Frankenhaeuser für wertvolle Hinweise, Dr. B. Folkow für die freundliche Überlassung eines Hand-Plethysmografen und E. Hansen für Hilfe bei der Beschaffung der Plethysmografen. Den Leitz-Werken in Wetzlar, insbesondere Herrn W. Freund, sind sie für entgegenkommende Unterstützung bei fotografischen Problemen sehr verbunden.

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Halide Transport in Red Blood Cells.

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Abstract.

TOSTESON, D. C. Halide transport in red blood cells. *Acta physiol. scand.* 1959. *46*. 19—41. — A flow tube for the measurement of rapid transport processes in red cells is described. The method is applicable to the measurement of transport processes with half-times between 0.05 and 1 sec in suspensions of cells which have an average diameter greater than 1 micron. The rate constants for outflux of Cl^{38}, Br^{82}, F^{18} and I^{131} from washed beef and human red cells suspended in chloride-phosphate medium at room temperature were measured under conditions of equilibrium of total halide in the system. The values of the rate constant were 3.1, 0.6, 0.3 and 0.06 sec^{-1} respectively for both beef and human cells. The ratios of cell to medium concentrations of the various halides at equilibrium were measured by isotope dilution and found to be 0.58, 0.63, 0.50 and 1.06 for Cl^{38}, Br^{82}, F^{18} and I^{131} respectiveiy in beef red cells. Similar ratios were observed in human cells. The rate constants for Cl^{38} outflux from beef red cells washed and suspended in bromide-phosphate and iodide-phosphate media were 1.8 and 0.6 sec^{-1} respectively, both much lower than the value of 3.1 observed in chloride-phosphate medium.

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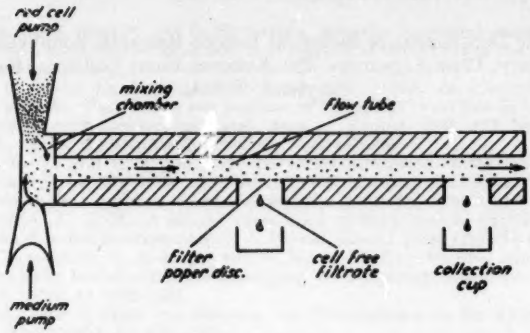

Fig. 1. This figure shows a schematic diagram of the flow tube apparatus used in these experiments.

While alkali cations penetrate the plasma membrane of red blood cells rather slowly, the negatively charged halide anions penetrate with great rapidity. Because of this high rate of transport, measurements of the kinetics of halide movement through the red cell membrane are technically difficult. This paper reports such measurements made with the use of a modification of the flow tube technique first used in physiological work by HARTRIDGE and ROUGHTON (19, 23). The results indicate that chloride ions traverse the surface of beef and human red cells one million times more rapidly than do potassium ions. Under standard conditions, the relative rates of transport of halide ions in beef red cells are $Cl > Br > F > I$. The effect of varying the concentrations of these ions on the transport rates is also reported.

Methods.

1. Flow tube apparatus.

In principle, the flow tube converts time to distance (see Fig. 1). Two pressure pumps, one containing red cells labelled with radioactive halide ions, the other containing non-labelled cell free medium, supply fluid to the mixing chamber at a constant flow rate. In the mixing chamber, the concentration of radioactive halide ions outside the cells suddenly falls, and the tracer begins to move out of the cells. This process continues during the flow of the diluted cell suspension down the tube. At intervals, the wall of the tube is replaced by filter paper which does not allow red cells to pass. Drops of the cell free filtrate emerging from these ports during steady flow are collected for analysis. From these measurements and a knowledge of the flow velocity, it is possible to calculate the amount of tracer emerging from the red cells during the time required for a volume element of the cell suspension to move from the mixing chamber to the filter.

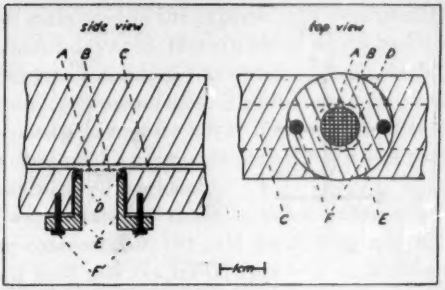

Fig. 2. This figure shows a scale drawing of the filters in the flow tube apparatus used in these experiments.

The symbols in the drawing refer to the following:

A: millipore filter;
B: supporting bolting cloth;
C: flow tube proper;
D: rubber O-ring;
E: Polymethacrylate plug;
F: brass screws.

The flow tube was made out of polymethacrylate. The tube itself consisted of a rectangular slit 1 cm wide, 1 mm thick and 33 cm long. At 5 cm intervals the wall of the tube was replaced by six circular discs of Millipore filter paper, each 1 cm in diameter (Fig. 2). This filter paper has a rather uniform pore diameter of 0.8 μ. Each filter was supported by a disc of bolting cloth and held in place by a rubber O ring which was compressed by a polymethacrylate plug. The plug was fastened to the tube by two brass screws. During flow the drops of filtrate emerged from the tube through the 1 cm hole in the center of this plug.

The mixing chamber was also constructed from polymethacrylate. The chamber was in the form of a flattened ellipse 12 mm long, 3 mm wide and 1.5 mm deep (see Fig. 3). The corners of the chamber were fed by four 18 gauge steel hypodermic needles. Flow from these needles into the chamber produced tangential mixing. The mixed cell suspension passed from the mixing chamber to the tube through an 8 mm by 0.8 mm slit in the polytetra fluorethylene gasket which separated the two parts. Four brass screws fixed the mixing chamber to the flow tube.

The needles of the mixing chamber were connected to four, 1 cm inner diameter, polyvinyl chloride tubes by means of steel adapters, the diameters of which smoothly decreased from 1 cm to 1 mm. Two of these polyvinyl chloride tubes were connected to a pressure pump containing red cells, while the other two tubes led to a pump containing cell-free suspension medium. Each pump consisted of a polyethylene bag enclosed in a 3 l glass pressure bottle. The bag was connected to the polyvinyl chloride tubing by a glass tube containing a 3-way stopcock through which the bag could be loaded, drained, and washed. The pressure bottle was connected to a compressed air source

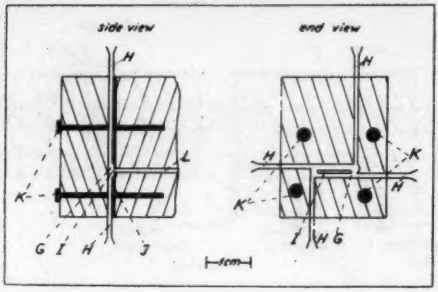

Fig. 3. This figure shows a scale drawing of the mixing chamber of the flow tube used in these experiments.

The symbols in the drawing refer to the following:

G: mixing chamber, proper;
H: No. 18 gauge needles;
I: slit from mixing chamber to flow tube;
J: Polytetrafluorethylene gasket;
K: brass screws;
L: flow tube proper.

by a reducing valve. A 24 gauge needle through the rubber stopper in the pressure bottle allowed for a slow constant flow of air through the pump and helped to stabilize the pressure. Constant pump pressures between 0 and 2 atm were obtainable with this system.

The filtrate emerging from the 6 Millipore discs was collected in polymethacrylate cups mounted in a sliding rack suspended beneath the flow tube. This rack contained twelve holes arranged in two rows of six. One row contained the collection cups while the other row contained six funnels drained by rubber tubing. During the early part of flow the filtrate was discarded through the funnels. This was necessary to avoid contamination of the filtrate with fluid present in the filters before the establishment of steady flow. The rack was then shifted so as to allow collection of samples for analysis. The volume of filtrate available for analysis varied between 0.1 and 0.4 ml depending on the pressure used, but duplicate 0.1 ml samples were usually obtainable. The total filtration rate through all 6 filters never exceeded 1 % of the flow rate.

The outflow from the tube was collected in a large funnel drained by a 2-way stopcock. In order to measure the flow rate, the outflow was collected in a graduated cylinder for a period of time measured by stop watch. Volume flow rates between 1.5 and 10 ml/sec (equal to flow velocities from 15 to 100 cm/sec) were readily measured in this way.

2. *Experimental Procedure.*

Both beef and human red cells were used. Beef blood was obtained from the abattoir using heparin as anti-coagulant. The blood was stored in the ice box without sterile precautions until

use. The beef cells used in the experiments were usually 1 day, but never more than 3 days old. Human blood which had been stored in acid-citrate-dextrose solution was obtained from the blood bank of the Rigshospital in Copenhagen. The human cells used in the experiments reported here were never more than 1 week old with the following exception. Experiments with I^{131} with Cl as carrier were done on human cells 2 weeks old.

The cells were washed 3 times in equal volumes of medium of the following composition: 151 mM NaCl, 3.35 mM KCl, 1.65 mM KH_2PO_4, and 9.35 mM Na_2HPO_4 (pH = 7.4). Following the last wash, a tracer amount of the halide isotope under study was added to the concentrated cell suspension. Tracer was added to ca. 200 ml of packed cells in the following forms in the various experiments: Cl^{38}, 4—8 ml of 0.17 M NaCl; Br^{82}, 0.1—1.0 ml, 1.4 M NH_4 Br; F^{18}, ca. 5 ml of 0.17 M NaCl or 0.17 M NaF which was used to wash out the container in which the isotope was made; I^{131}, a tracer quantity of NaI^{131} dissolved in 5—10 ml 0.17 M NaCl or 0.17 M NaI. After mixing for a few minutes, this concentrated cell suspension was placed in the cell pump. The cells were injected into the pump bag and allowed to run back out through the 3-way stopcock into the injecting syringe several times in order to ensure adequate mixing in the cell pump. A sample of the concentrated cell suspension in the pump was then removed for analysis. Medium identical to that in which the cells were washed, except that it was free of tracer, was placed in the medium pump. All experiments were done at room temperature (21—25° C). After the run, samples of the outflow and the several filtrates were also taken for analysis.

Hematocrit determinations were made on samples from the cell pump and the outflow from the tube. In early experiments these measurements were made in clinical hematocrit tubes spun at 3,000 × g for 30 min. Later, they were made in capillary tubes centrifuged at 16,000 × g for 20 min. The tubes were read with a cathetometer. The cell pump and outflow samples were then centrifuged and the supernatant medium analyzed for radioactivity, and, in most cases hemoglobin. The hemoglobin measurements were made with a Beckman model B spectrophotometer at a wave length of 405 mμ.

All radioactivity determinations were made on dried aliquots which had been pipetted into planchets containing lens paper to assure even distribution. The planchets were then counted under

a G-M tube. The standard error of the counting rate was usually kept within 3 %. The sources of the isotopes used were as follows: F^{18} and Cl^{38} were made in the cyclotron of the Institute for Theoretical Physics in Copenhagen. F^{18} was produced carrier-free by a d-α reaction from Ne, while Cl^{38} was made by bombardment of NaCl. The Cl^{38} was separated from the Na^{24} produced simultaneously by distillation in strong acid. Br^{82} and I^{131} were obtained from Atomic Energy Research Establishment, Harwell, England.

3. Analysis of results.

The concentration of tracer in the medium in the mixing chamber, R_o, was calculated as follows:

$$R_O = \left[\frac{\gamma_E\,(1-\gamma_p)}{\gamma_p\,(1-\gamma_E)}\right] R_p \qquad (1)$$

where γ_p and γ_E represent the hematocrit in cell pump and in the outflow from the tube respectively, and R_P is the concentration of tracer in the medium used in the cell pump. In many experiments in which hemoglobin was placed in the medium in the cell pump, R_o was also calculated from the relation

$$R_O = \left[\frac{Hb_E}{Hb_p}\right] R_p \qquad (2)$$

where Hb_p and Hb_E represent the hemoglobin concentration in the medium in the cell pump and the outflow respectively. In early experiments when the hematocrits were spun at $3000 \times g$ the R_o value calculated from hemoglobin dilution exceeded that calculated from the hematocrits. In the latter experiments when high speed hematocrits were used, the two values agreed well (Table I).

The ratio of cell to medium concentration of tracer halide at equilibrium, r, was calculated from the following relation,

$$r = \frac{\left(\frac{1-\gamma_E}{\gamma_E}\right) R_E - \left(\frac{1-\gamma_p}{\gamma_p}\right) R_p}{R_p - R_E} \qquad (3)$$

where R_E is the concentration of tracer in the medium in the outflow from the tube, *i. e.* the new equilibrium value. This equation assumes that the equilibrium distribution ratio of the tracer is not influenced by passage of the cell suspension through the tube. This assumption was shown to be valid by direct analysis of cells and medium (see Table II).

Table I.

Test of hemolysis and mixing in flow tube.

Hematocrit Method	Hemoglobin Concentration Observed		Hemoglobin Concentration Calculated from Hematocrit
	First Port	Outflow	First Port and Outflow
Slow Spin	0.207	0.210	0.179
Fast Spin		0.154	0.150

All values in the table are expressed as optical density at 405 mM. Values obtained with both slow and fast centrifugation are from single experiments which are typical of 4 yielding similar results. The values in the right hand column were calculated from measurements of hemoglobin in the medium from the cell pump and of hematocrit in the cell pump and outflow by equation *1*.

The rate constant for halide outflux, k_o, was obtained from the following equation,

$$k_o = \frac{0.693}{T}\left[\frac{1-\gamma_E}{1-\gamma_E\,(1-r)}\right] \tag{4}$$

where T is the half-time for passage of the tracer halide from one equilibrium state to another. The half-time was obtained graphically from the relation

$$\frac{\delta}{\delta t}\left[\ln\left(\frac{R_E-R_t}{R_E-R_o}\right)\right] = \frac{0.693}{T} \tag{5}$$

where R_t is the concentration of tracer in the medium at time t. In all experiments, the function on the left hand side of equation *5* could be described as a single exponential function of time. However, in some cases (*e. g.* F^{18}, Br^{82}, I^{131}), the extrapolation of the function on the left hand side of equation *5* to zero time did not yield a value of unity. In none of these cases could a rapid component in the exchange process be resolved. Therefore, the outward rate constant in these experiments was calculated by a modification of equation *4*. This modification assumes that the cell halide comprises two compartments, one of which exchanges with the halide in the medium instantaneously as the suspension reaches the mixing chamber, the other of which exchanges more slowly during flow of the suspension down the tube. Under these circumstances k_o was calculated from the relation:

$$k_o = \frac{0.693}{T}\left[\frac{1-\gamma_E}{1-\gamma_E+\gamma_E\,(r-r_F)}\right] \tag{6}$$

Table II.

Test of calculation of equilibrium Cl^{38} distribution.

Cell Type	Ratio of Cell to Medium Cl^{38} Concentration		
	Observed		Calculated
	Cell Pump	Outflow	
Beef.....	0.49	0.51	0.54
Man	0.55	0.54	0.55

The values in the table represent the ratio of cell Cl^{38} expressed as cpm/l cells, to medium Cl^{38} in cpm/l medium. The results are from single experiments. The values in the observed columns were obtained by measuring the Cl^{38} in cells and medium from cell pump and outflow. The values in the right hand column were calculated from measurements of Cl^{38} and hematocrit in medium from cell pump and outflow by equation *3*.

where r_F represents the ratio of the concentration of rapidly exchanging halide in the cells (mmoles rapidly exchanging halide/l of *all* cells) to the halide concentration in the medium. r_F was calculated from the following equation:

$$r_F = \frac{\left(\frac{1-\gamma_E}{\gamma_E}\right) R'_o - \left(\frac{1-\gamma_P}{\gamma_P}\right) R_P}{R_P - R_E} \qquad (7)$$

where R'_o represents the concentration of tracer halide in the medium after equilibration with the rapidly exchanging cell halide in the mixing chamber, but before exchange with the slowly exchanging halide has begun. The value of R'_o was calculated by extrapolation of the function $\left[\frac{R_E - R_t}{R_E - R_o}\right]$ to zero time. From the value of the intercept I, R'_o was calculated from the relation

$$R'_o = R_E - I\,(R_E - R_o). \qquad (8)$$

If the intercept equals unity, value of R'_o calculated from equation *8* equals the value of R_o calculated from equation *1* and there is no evidence for a rapid compartment.

Results.

1. Tests of the performance of the flow tube.

The adequacy of mixing was tested by adding a small amount of hemoglobin solution to the concentrated suspension in the cell pump. If mixing in the mixing chamber were perfect, the hemoglobin concentration in the filtrate from the first port after

the mixing chamber should equal that in the medium of the suspension flowing out of the tube. Table I shows that this condition obtained in experiments where the flow velocity varied from 17 was to 74 cm/sec. A further test of mixing is that the dilution in the mixing chamber of red cells and a non-penetrating solute like hemoglobin have similar characteristics. This may be evaluated by comparing the observed hemoglobin concentration in first port filtrate and outflow medium with the concentration calculated from measurements of hemoglobin in medium from the cell pump and hematocrit in cell pump and outflow by equation *1*. In our early experiments, when the hematocrit was estimated by relatively slow centrifugation, 3,000 $\times$ *g*, the calculated value was invariably about 20 % below the observed concentration (Table I). In later experiments, when the hematocrits were measured by centrifugation at 16,000 $\times$ *g*, the two values usually agreed well.

The method of calculation of the halide concentration ratio between cells and medium (equation 3) was evaluated by direct measurement of the concentration of tracer halide in cells and medium from cell pump and outflow. There was good agreement between the observed and calculated values for Cl^{38} ratios in beef and human red cells (Table II). As will be discussed later, this was not true for F^{18}.

2. Chloride transport.

a) The distribution and kinetics of Cl^{38} in the standard chloride system.

Table III shows that the mean ratios of cell to medium Cl^{38} concentration in beef red cells was 0.58 and in human red cells 0.54. These values may be compared with the value of 0.56 obtained by HARRIS and MAIZELS (1952) for human red cells and 0.52 by SHAW (1955) for horse red cells, both suspended in phosphate buffer at pH 7.4. In most experiments there was no evidence of a rapidly exchanging cell fraction of Cl^{38}, hence the r_F for this isotope was close to zero.

Cl^{38} was found to disappear from labelled beef red cells by a first order process with a half-time of about 0.2 sec, *e. g.* 0.22 sec in the typical experiment shown in Fig. 4. The rate constant for Cl outflux, k_o calculated from the half-time by equation *4*, was 2.8 $(sec)^{-1}$ in this experiment. Reference to Table III shows that this value was at the lower end of the range observed in five experiments with beef red cells. The mean value of k_o for beef red cells was

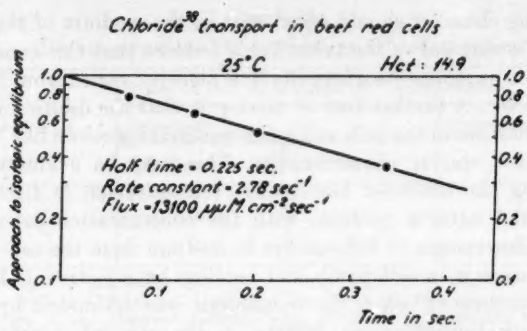

Fig. 4. This figure shows a typical experiment with beef red cells labelled with Cl^{36}. The approach to isotopic equilibrium, $\frac{(R_e - R_i)}{(R_e - R_o)}$ from equation 5 in the text, is plotted on the ordinate, as a function of time in seconds on the abscissa.

3.1 sec^{-1}. The chloride flux was calculated from k_o by the relation,

$$M = \frac{k_o\, r\, C_m}{S} \qquad (9)$$

where M is the flux in $\mu\mu$moles cm^{-2} sec^{-1}, r is the ratio of cell to medium chloride concentration, C_m is the concentration of chloride in the suspension medium in $\mu\mu$M, and S is the surface to volume ratio of the red cells in cm^2 l^{-1}. The value of S was calculated to be 1.96×10^7 for beef red cells from measurements of Ponder and Saslow (1930). Thus, in the experiment shown in Fig. 4 with beef red cells suspended in a medium containing 154 mmoles/l Cl the flux was 13,100 $\mu\mu$moles cm^{-2} sec^{-1}.

A single measurement of Cl^{36} movement in human red cells yielded values for rate constant and chloride flux which are similar to those observed in beef red cells (Fig. 5). The half-time in this experiment, 0.21 sec, may be compared with the value of 0.2 sec, and 0.14 sec, observed by Dirken and Mook (1931) and Luckner (1939) respectively, for the decay of a chloride concentration gradient in human red cells.

b) The effects of chloride concentration and other variables on Cl^{36} distribution and kinetics.

Several experiments were done to define further the characteristics of chloride transport in beef red cells. One variable which

Table III.

Distribution and transport of tracer halides in red cells.

Cell Type	Isotope	Equilibrium Ratio of Cell to Medium Isotope Concentration			Outward Rate Constant Slow Fraction $(sec)^{-1}$
		Fast Fraction	Slow Fraction	Total	
Beef.....	Cl^{38}	0.06 (0.00—0.17)	0.52 (0.38—0.61)	0.59 (0.38—0.79)	3.1 (2.6—3.7)
Man.....	»	0.00	0.54	0.54	3.1
Beef.....	Br^{82}	0.16 (0.09—0.23)	0.47 (0.35—0.61)	0.63 (0.44—0.76)	0.6 (0.5—0.8)
Man.....	»	0.08 (0.00—0.15)	0.55 (0.43—0.59)	0.63 (0.43—0.72)	0.6 (0.4—0.9)
Beef.....	F^{18}	0.05 (0.00—0.09)	0.45 (0.40—0.52)	0.50 (0.40—0.61)	0.3 (0.2—0.4)
Beef.....	I^{131}	0.16 (0.00—0.25)	0.90 (0.73—1.15)	1.06 (0.94—1.25)	0.06 (0.01—0.08)
Man.....	»	0.12 (0.09—0.18)	1.09 (0.81—1.47)	1.21 (0.90—1.65)	0.07 (0.04—0.12)

The large numbers above represent the means of the following numbers of experiments: Cl^{38}-beef, 5; Cl^{38}-man, one; Br^{82}-beef, 3; Br^{82}-man, 4; F^{18}-beef, 3; I^{131}-beef, 6; I^{131}-man, 4. The small numbers in parentheses represent the range. In all experiments, chloride was the carrier halide in the system. All experiments were done at room temperature, ca. 23° C.

is likely to be important in determining the rate of Cl^{38} movement is the total concentration of chloride in the system. In order to evaluate this factor, beef red cells were washed in medium identical to that described in the section on *Methods*, except that the NaCl was replaced by NaBr or NaI. In the case of the experiments with Br, the final Cl concentration in the cells after addition of the small amount of carrier chloride associated with the Cl^{38} was 7.8 mM. In the experiments with I, the corresponding figure was 12.3 mM. In both cases, the medium pump contained the solution in which the cells had been washed which contained 3.3 mmoles/l Cl. Thus, the Cl concentration difference across the cell membrane produced by addition of $NaCl^{38}$ to the cells was about 4 mM in the Br and 9 mM in the I experiments. Measurements of Cl^{38} distribution and kinetics under these conditions are summarized in Table IV. Although the equilibrium distribution of Cl^{38} was not affected, the presence of both Br and I produced a variable but definite fraction of rapidly exchanging Cl^{38}. Thus r_F was 0.09 for the Br and 0.13 for the I system. Br reduced the value of k_o for Cl^{38} from 3.7 to 1.8 sec^{-1}, while I slowed Cl^{38} movement

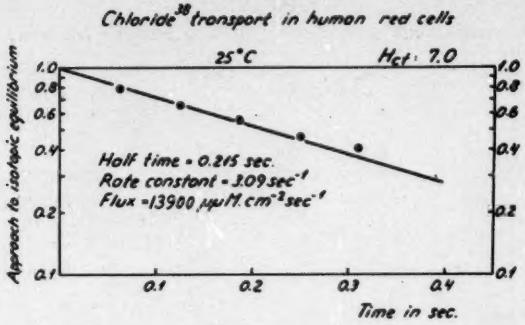

Fig. 5. This figure shows a typical experiment with human red cells labelled with Cl^{36}. The approach to isotopic equilibrium, $\frac{(R_e - R_t)}{(R_e - R_o)}$ from equation 5 in the text, is plotted on the ordinate as a function of the time in seconds on the abscissa.

to one fifth the rate observed when Cl was the only halide in the system. This slowing of Cl movement by Br and I may be related to the effects of I on muscle (see discussion).

One of the most impressive properties of the red cell membrane is its permselectivity, that is, its high permeability to anions as compared with cations. It is reasonable to suppose that this permselectivity depends on the presence of positively charged groups in the channels through which the chloride ions diffuse. A few experiments aimed at defining the nature of these hypothetical groups were done. The rate constant for Cl outflux was found to be the same at pH 7.3 and 7.6. Thus, the hypothetical positive groups in the membrane did not appear to be titrated in this pH range. The whole cell was titrated since the chloride concentration ratio fell from 0.58 at the lower to 0.40 at the higher pH, as expected. Furthermore, addition of imidazole diphosphate and amino phosphate[1], compounds which react with primary amino groups (ROSENBERG 1956), had no effect on chloride transport in beef red cells.

3. Bromide transport.

a) Distribution and kinetics of Br^{82} in the standard chloride system.

The equilibrium ratio Br^{82} concentration in cells to that in medium was 0.63 for both beef and human red cells (Table III). This value does not differ significantly from that observed for

[1] These substances were kindly given to me by T. ROSENBERG.

Table IV.

Effect of carrier on tracer halide transport.

Cell Type	Isotope	Carrier	Equilibrium Ratio of Cell to Medium Isotope Concentration			Outward constant slow fraction $(sec)^{-1}$
			Fast Fraction	Slow Fraction	Total	
Beef..	Cl^{38}	Cl	0.06	0.52	0.58	3.1
		Br	0.09 (0.05—0.13)	0.50 (0.49—0.52)	0.59 (0.54—0.65)	1.6 (1.3 —2.0)
		I	0.13 (0.00—0.27)	0.44 (0.43—0.45)	0.57 (0.45—0.70)	0.6 (0.5 —0.7)
Beef..	Br^{82}	Cl	0.16	0.47	0.63	0.6
		Br	0.04 (0.00—0.07)	0.57 (0.42—0.74)	0.61 (0.42—0.81)	0.8
Beef..	F^{18}	Cl	0.05	0.45	0.50	0.3
		F	0.07	0.45	0.52	0.8
Man..	I^{131}	Cl	0.12	1.09	1.21	0.07
		I	0.23 (0.16—0.31)	0.92 (0.87—0.97)	1.15 (1.03—1.28)	0.03 (0.03—0.03)

The large values in the table represent the mean from the following numbers of experiments: Cl^{38}-Br carrier, two; Cl^{38}-I carrier, two; Br^{82}-Br carrier, two for concentration ratios but only one for rate constant; I^{131}-I carrier, two; and a single experiment with F^{18}-F carrier. The values for all isotopes with Cl as carrier are reproduced from Table III for ease of comparison. The small numbers in parentheses represent the range. All experiments were done at room temperature, ca. 23° C.

Cl^{38}. It may be compared with the figure 0.56 obtained for the Br^{82} concentration ratio in dog blood by HASTINGS *et al.* (1931). In a number of experiments with Br^{82} a rapidly exchanging fraction of cell bromide was observed. The concentration ratios for fast and slow fractions were calculated to be $r_F = 0.16$ and $r_S = 0.47$ for beef red cells.

The rate constant for bromide outflux was found to be substantially smaller than the value observed for chloride in both beef and human cells (Table III). A graphical comparison of a typical experiment with Cl^{38} and Br^{82} in beef cells can be seen in the bottom two curves of Fig. 6.

b) The effect of bromide concentration on Br^{82} distribution and kinetics.

The effect of concentration of bromide on bromide flux was evaluated by replacing with NaBr the NaCl in the suspension medium used to wash the beef red cells. After addition of the

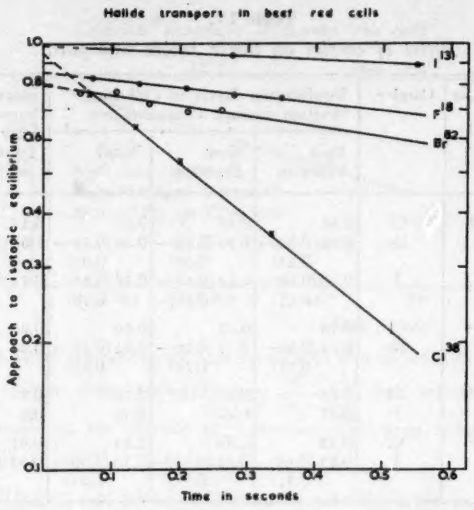

Fig. 6. This figure shows typical experiments with beef red cells labelled with Cl^{36}, Br^{82}, F^{18} and I^{131}. The approach to isotopic equilibrium, $\frac{(R_e - R_t)}{(R_e - R_0)}$ from equation *5* in the text, is plotted on the ordinate as a function of the time in seconds on the abscissa.

carrier Br necessarily associated with the NH_4 Br^{82}, medium associated with the packed cells in the cell pump contained 147 mmoles/l Br, 3.4 mmoles/l Cl and 11 mmoles/l H PO_4—H_2PO_4. The medium in the medium pump was identical except that the Br concentration was 133 mM. The results of measurement of the rate constant for bromide outflux in this system are summarized in Table IV. Clearly, raising the bromide concentration from a low value (1—4 mM in the experiments with chloride as carrier) to a 133 mM solution of NaBr has relatively little effect on bromide distribution or rate constant in beef red cells. In other words, the bromide flux is approximately proportional to the concentration of bromide over the range studied. The bromide flux in the experiment with Br as carrier was 3,100 $\mu\mu$moles cm^{-2} sec^{-1} as compared with the value of 13,100 for Cl flux with chloride as carrier.

4. Fluoride transport.

a) The distribution of F^{18} and the kinetics of F^{18} movement in the standard chloride system.

The distribution ratio of F^{18} in beef red cells as calculated by equation *3* was 0.50, somewhat lower than the values obtained for Cl^{38} and Br^{82} (Table III). A small and variable fast fraction which yielded a figure for $r_F = 0.05$ was also observed. However, the interpretation of these calculated ratios is made difficult by the fact that they did not agree with the measured concentration ratio in cell suspension from the outflow (mean 0.75, range 0.65—0.80). Agreement was much better between the calculated ratio and the ratio observed in the cell suspension in the cell pump (0.54 in two experiments). The system behaved as if a certain amount of the F^{18} became bound in the cells while they were in the cell pump, and failed to come out during the run through the tube. This interpretation is supported by the fact that substitution of F for Cl as carrier halide in the system reduced the discrepancy between calculated and measured ratios.

The rate constant for F^{18} outflux for beef red cells in the standard chloride system was 0.3 sec^{-1}, substantially slower than the value for Br^{82} and only about 1/9 the value for Cl^{38} (Fig. 6).

b) The effect of concentration on fluoride distribution and transport.

A single experiment was done in which fluoride replaced chloride as carrier halide in beef red cells. After washing and addition of F^{18}, the concentrations in the medium associated with the packed cells in the cell pump was 127 mM F, 9 mM Cl and 11 mM PHO_4—H_2PO_4. The composition of the medium in the medium pump was identical. The calculated distribution ratios for F^{18} were not affected by this change in concentration (Table IV). However, agreement between the calculated and observed ratios was improved when fluoride was the major carrier halide in the system. Thus, the ratio of cell to medium F^{18} concentration observed in the cell pump and outflow were 0.65 and 0.58 respectively, as compared with a calculated value of 0.52. Substitution of fluoride for chloride increased the rate constant for fluoride outflux from 0.3 to 0.8 sec^{-1}. The fluoride flux with fluoride as carrier was 2,300 $\mu\mu$moles cm^{-2}. l^{-1}.

5. Iodide transport.

a) Distribution and kinetics of I^{131} in the standard chloride system.

The ratio of I^{131} concentration in cells to that in the medium was greater than unity, 1.06 in beef and 1.21 in human red cells (Table III), confirming the observations of OWEN and POWER (1953). In both cell types, rapidly exchanging iodide fractions were observed which yielded r_F values of 0.16 and 0.12 for beef and human red cells respectively.

Fig. 6 shows clearly that I^{131} movement was slower than any of the other halides in beef red cells. The value of k_o was 0.06 for beef and 0.07 for human cells (Table III).

b) The effect of iodide concentration on I^{131} distribution and kinetics.

In two experiments, human cells were washed with medium in which 50 mmoles/l NaI had replaced 50 mmoles/l NaCl. After addition of a tracer quantity of I^{131}, these cells were placed in the cell pump, while the medium pump contained the iodide wash medium. This procedure did not significantly affect the distribution or the kinetics of I^{131} in this system. We may conclude that the rate constant for iodide outflux is independent of iodide concentration in the range from 1 mmole/l to 50 mmoles/l.

Discussion.

A consideration of the magnitude of the chloride flux measured in these experiments reveals several interesting properties of the surface membrane of beef and human red cells. Considered in relation to the ionic permeability of other cells, and to the rate of cation transport in red cells, the chloride flux through the membrane is very high. On the other hand, viewed in relation to the rate of chloride movement in an equivalent layer of 0.1 N KCl, or to the water flux in red cells, the membrane is quite impermeable to chloride.

The chloride flux across both beef and human red cells membranes is about 1.3×10^4 $\mu\mu$moles cm^{-2} sec^{-1}, about 10 times the Na flux in active Sepia axons, about 100 times the K outflux

from resting sepia fibers, and about 1,000 times the K outflux in resting frog muscle (HODGKIN 1951). Even when compared with tissues specialized for the secretion of chloride containing solutions, the rapidity of chloride movement in red cells is evident. Thus, the chloride flux from mucosal to the serosal surface of the isolated, short-circuited frog gastric mucosa bathed in Ringer's solution is 1.8×10^3 $\mu\mu$moles cm^{-2} sec^{-1} (HOGBEN 1955), while in the isolated frog skin the chloride outflux is 14—140 $\mu\mu$moles cm^{-2} sec^{-1} (depending on the electrical potential and concentration, KOEFOED-JOHNSEN *et al.* 1952), both much smaller than the value observed in red cells.

The relative rapidity of chloride movement across the red cell membrane is most vividly illustrated by comparison with the rate of cation transport in this same cell system. SHAW (1955) has found that the rate constant for K outflux in beef red cells is 1.8×10^{-6} sec^{-1}, less than one millionth of the rate constant for chloride movement of 3.1 sec^{-1} in these cells. This degree of permselectivity compares favourably with that observed in positively charged, artificial ion exchanger membranes (SOLDANO and BOYD 1953 a).

In view of this high degree of permselectivity, calculation of that part of the electrical resistance of the red cell surface which is due to chloride ions should provide a good estimate of the total electrical resistance of the membrane. The chloride resistance, R_{Cl}, can be calculated from the relation

$$R_{Cl} = \frac{RT}{F^2 M_{Cl}} \qquad (10)$$

where R is the gas constant, T is the temperature, F is Faraday's number, and M_{Cl} is the chloride flux (USSING 1955). For beef cells with a flux of 1.3×10^4 $\mu\mu$moles cm^{-2} sec^{-1} the chloride resistance is 19 ohms cm^2. Unfortunately, no good measure of the total electrical resistance of the red cell membrane is available. Estimates have varied from 0.1 ohms cm^2 (MCCLENDON 1926) to an infinite resistance (FRICKE 1933). Therefore, it is not possible to confirm the relation between the chloride resistance and the total electrical resistance of the membrane. If we assume that the thickness of the red cell membrane is 100 Å, we may calculate that the specific chloride resistance of the membrane is 19×10^6 ohm cm. This may be compared with the specific chloride resistance in 0.1 N KCl at 25° C of 154 ohm cm (HARNED and OWEN 1950). If we assume

that this marked increase in chloride resistance in the membrane is due to a reduction in the area available to the ions, we may surmise that only 8×10^{-6} times the total area is involved in chloride movement. Even if we assume that the specific chloride conductance in the membrane is 10^{-3} times the value in 0.1 N KCl, only one part in one hundred and thirty of the membrane area would be necessary to account for the observed chloride flux. Thus, the apparent rapidity of chloride transport in red cells is in large measure due to the geometry of the system (*i. e.* the extreme thinness of the membrane, and the small size of the cells) rather than to any inherent speed of the transport process itself.

This restriction to chloride movement imposed by the membrane is further emphasized by comparing the half-time for Cl^{38} equilibration in beef red cells of about 0.2 sec with half-time of 0.0031 sec (comprising about 50 % of the system) and 0.030 sec (the remaining 50 %) for DHO equilibration in suspensions of the same cells at a comparable temperature and hematocrit (TOSTESON 1959). The half-time for THO equilibration in human red cells is 0.0036 sec (PAGANELLI and SOLOMON 1956). Thus, in both cell types, water traverses the cell membrane about 70 times faster than chloride.

The results reported here do not define unequivocally the mechanism of halide transport across the red cell membrane. Active transport of halide is ruled out by the fact that the ions appear to be distributed at thermodynamic equilibrium. However, it is not so easy to define the relative roles of passive diffusion and exchange diffusion (USSING 1955), in halide transport in red cells. Certain characteristics of chloride transport are consistent with transport by passive diffusion. These include: 1) The half-time for decay of a concentration difference of isotopic chloride reported here is about the same as that for the dissipation of an electrochemical potential difference for total chloride exchanging with bicarbonate (DIRKEN and MOOK 1931, LUCKNER 1939). 2) The Q_{10} (24–38° C) for chloride-bicarbonate exchange is 1.2 (LUCKNER 1948). On the other hand, some aspects of halide transport require further study before they can be accepted as consistent with passive diffusion. These include: 1) There are large differences in the rates at which the various ions traverse the red cell membrane. Our finding that Cl penetrates much faster than I confirms the conclusions of ØRSKOV (1934) but disagrees with the results

of MAIZELS (1934) who was probably measuring the relative distribution ratios rather than the relative permeabilities of the rapidly moving halides (although his method was valid for measuring the kinetics of slow anion exchanges). The ratio of Cl to I rate constant reported here for red cells is much greater than the ratio observed in artificial anion exchangers (SOLDANO and BOYD 1953 a). 2) Cl transport is markedly inhibited by Br and I. Because of the relative impermeability of the cells to cations, practically all of the anion movement across the membrane must occur by a process of anion exchange. Thus, the rate at which Cl emerges from the cell is partly dependent on the rate at which another anion can enter. The fact that Br and I move through the red cell membrane more slowly than Cl may account for the reduced Cl rate constant in cells equilibrated with these ions. This may be analogous to the reduction in the self-diffusion coefficient of a rapidly moving ion in an ion exchange when a slowly moving ion is present in the exchanger (SOLDANO and BOYD 1953 b). However, an effect of Br or I on an element of Cl exchange diffusion cannot be ruled out at present.

It is interesting to note that the inhibition of chloride transport by other ions is not confined to red cells, since the outflux of Cl^{36} from frog muscle is inhibited by nitrate (ADRIAN 1957). It is possible that this phenomenon is related to the marked prolongation of the active state by nitrate and I in whole skeletal muscle (KAHN and SANDOW 1950, HILL and MACPHERSON 1954), and in single fibres (HOROWICZ and HODGKIN 1956).

In conclusion, it is probable that halide transport across the beef and human red cell membrane occurs by a process of passive diffusion. However, certain unusual features of the halide transport process in these systems (*e. g.* the selectivity for different halides, effect of I on Cl transport) must be studied further before an element of halide exchange diffusion can be definitely excluded.

Summary.

1. A flow tube for the measurement of rapid transport processes in red cells is described.

2. The rate constants for the outflux of Cl^{38}, Br^{82}, F^{18}, and I^{131} from washed beef and human red cells suspended in chloride-phosphate medium at room temperature were measured under

conditions of electrochemical equilibrium of total halide in the system. The values of the rate constants for beef cells were 3.1, 0.6, 0.3, and 0.06 sec^{-1} respectively. The values for human cells were not significantly different.

3. The ratios of cell to medium concentrations of the various halides at equilibrium were measured by isotope dilution in both cell types. The ratios for Cl^{38}, Br^{82}, F^{18}, and I^{131} were 0.58, 0.63, 0.50, and 1.06 respectively in beef cells. The ratios for human cells were about the same.

4. The rate constants for Cl^{38} outflux from beef red cells washed and suspended in bromide-phosphate and iodide-phosphate media were 1.8 and 0.6 sec^{-1} respectively. Thus bromide and iodide reduced the rate constant for Cl^{38} outflux to one half and one fifth the value observed in chloride medium.

I wish to thank Prof. Ussing, who suggested the flow tube approach to me during a conversation in America in 1952, and who provided space in his laboratory, and advice and encouragement during the progress of the work. My thanks are also due to Dr. R. Bowman of the National Heart Institute in Bethesda, Maryland, for help in constructing the flow tube and to Dr. Lassen who made the F^{18} and Cl^{38} in the cyclotron of the Institute for Theoretical Physics in Copenhagen.

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Appendix.

Symbols and definitions: .

R_p = Concentration of tracer in medium of cell pump.
R_o = » » » » » » mixing chamber.
R_e = » » » » » » outflow.
C_m = » » total halide in medium.
C_c = » » » » » cells.
C_{cf} = » » rapidly exchanging halide in cells.
r = C_c/C_m.
r_f = C_{cf}/C_m.
γ_p = hematocrit in cell pump.
γ_e = » » outflow.
C_m^* = $C_m\,(1-\gamma_e)$.
C_c^* = $\gamma_e C_c$.
C_{cf}^* = $\gamma_e C_{cf}$.
M^* = flux of halide between cells and medium per unit volume of cell suspension.
M_f^* = flux of halide between slowly exchanging cell fraction and medium per unit volume cell suspension.
M = flux per unit area of cell membrane.
t = time.
T = half-time for equilibration of tracer between cells and medium.

1° For a two compartment closed system in the steady state,

$$ln\left[\frac{R_e - R}{R_e - R_o}\right] = -(k_i + k_o)\,t$$

where $k_i = \dfrac{M^*}{C_m^*}$ and $k_o = \dfrac{M^*}{C_c^*}$ (Robertson 1957)

2° a) $k_i = \dfrac{M^*}{(1-\gamma_e)\,C_m}$ b) $k_o = \dfrac{M^*}{\gamma_e\, r\, C_m}$

3° $$k_i = \left[\frac{\gamma_e\, r}{1-\gamma_e}\right] k_o$$

4° Substituting 3° in 1°,

$$ln\left[\frac{R_e - R}{R_e - R_o}\right] = -k_o\left[\frac{1-\gamma_e(1-r)}{1-\gamma_e}\right]t$$

5° $$ln\ {}^1/_2 = -k_o\,\frac{1-\gamma_e\,(1-r)}{1-\gamma_e}\,T$$

6° $$k_o = \frac{0.693}{T}\left[\frac{1-\gamma_e}{1-\gamma_e\,(1-r)}\right]$$, equation 4 in the text.

7° For case in which cell halide comprises two fractions, slow (*S*) and fast (*F*), with the latter always in isotopic equilibrium with the medium,

$$ln\left[\frac{R_e - R}{R_e - R_o'}\right] = -(k_{is} + k_{os})\,t$$

where R_o' is the concentration of tracer in the medium of the mixing chamber after equilibration of fast fraction but before equilibration with slow fraction has begun.

$$k_{is} = \frac{M_s^*}{C_m^*} \qquad k_{os} = \frac{M_s^*}{C_c^* - C_{cf}^*}$$

8° $R_o' = R_e - I\,(R_e - R_o)$, equation *8* in text

where $I = \left[\dfrac{R_e - R}{R_e - R_o}\right] t = o$

and R_e is defined by equation *1* in text.

9° a) $k_{is} = \dfrac{M_s^*}{(1-\gamma_e)\,C_m}$ b) $k_{os} = \dfrac{M_s^*}{\gamma_e\,C_m\,(r-r_f)}$

10° Since R_b does not change during equilibration of fast fraction,

a) $R_b = \gamma_e\, r\, R_p + \dfrac{\gamma_e\,(1-\gamma_p)}{\gamma_p}\, R_p$

b) $R_b = \gamma_e\,(r-r_f)\,R_p + \gamma_e\, r_f\, R_e' + (1-\gamma_e)\, R_e'$

11° Solving 10° a, b simultaneously with *3* in text

$$r_f = \frac{\left[\dfrac{1-\gamma_e}{\gamma_e}\right] R_e' - \left[\dfrac{1-\gamma_p}{\gamma_p}\right] R_p}{R_p - R_e}, \text{ equation } 7 \text{ in text.}$$

12° Substituting 9° into 7°,

$$ln\left[\frac{R_e - R}{R_e - R_e'}\right] = -\,k_{os}\left[\frac{1-\gamma_e+\gamma_e\,(r-r_f)}{1-\gamma_e}\right] t.$$

13° $k_{os} = \dfrac{0.693}{T}\left[\dfrac{1-\gamma_e}{1-\gamma_e+\gamma_e\,(r-r_f)}\right]$, equation *6* in text.

14° $M = \dfrac{M^*}{\gamma_e\, S}$ where S is the surface to volume ratio for red cells.

15° Substituting 14° into 2° b, or 9° a,

a) $M = \dfrac{k_o\, r\, C_m}{S}$, equation *9* in text,

b) $M_s = \dfrac{k_{os}\,(r-r_f)\,C_m}{S}$

In the text the symbols k_o and M are used to refer to the rate constant and flux of the slow fraction whether or not it comprises the whole of the cell halide.

Acta physiol. scand. 1959. 46. 42—54.

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Quantitative Aspects of the Elimination of Neutral Violet by the Stomach.

By

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Abstract.

BILSKI R. and K. J. ÖBRINK. Quantitative aspects of the elimination of neutral violet by the stomach. *Acta physiol. scand.* 1959. *46*. 42—54. — It was shown in Heidenhain dogs that neutral violet was readily excreted from the blood. This excretion depended on the secretion rate of the gastric juice. Through experiments on isolated stomachs from mice it appeared probable that the elimination of the dye was due to a partition depending on the pH of the two sides of the mucosal membrane. At equal pH on both sides of the mucosa the final concentrations of the dye became equal on both sides. Part of the dye was captured in the mucosa itself, apparently depending on the state of activity of the mucosa. It is suggested that the accumulation of neutral violet in the gastric juice of dogs could also be a pH partition.

It is a well-known fact that many dyes are easily excreted through the gastric mucosa. Regarding the elimination of neutral red, the literature is already large and the reader is referred to

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papers by DAWSON and IVY (1925), MATSOU (1934—35), INGRAHAM and VISSCHER (1935), VISSCHER (1942) and KOLM, KOMAROV and SHAY (1945). Several comparative studies of the abilities of different dyes to enter the gastric juice may be found in these papers.

A general finding has been that dyes having basic properties are more readily excreted than others, and attempts to correlate this excretion to the pKa of the dye have been attempted (INGRAHAM and VISSCHER (1935), RAY and JUNG (1951), WOISLAWSKI (1952, 1953) and RAY and PEASE (1957)).

Several authors have correlated the appearance of dye in the stomach with an intact hydrochloric acid secretion. In the case of neutral red elimination such an acid secretion no doubt seems necessary. On the other hand the dye could be extracted from an achylic mucosa by instilled hydrochloric acid (HALLÉN 1949).

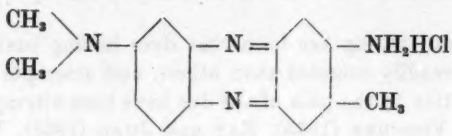
Only few papers have been concerned with the quantitative aspects of the elimination of dyes and almost none with its relation to the exact secretory state of the stomach. On the other hand several other components or drugs which have not the properties of dyes have been used in the study of gastric excretion and subjected to quantitative analyses. Thus COOKE, DAVENPORT and GOODMAN (1941) and DAVENPORT (1942) investigated the excretion of sulfonamides and SHORE, BRODIE and HOGBEN (1957) the excretion of various types of other drugs (see discussion).

In 1948, ÖBRINK found a very close relationship between the excretion of neutral red and the secretion rate (ml/min) of the gastric juice. By using a continuous intravenous injection technique for administration of the neutral red he considered that the blood concentration attained a constant steady level. When keeping the injection rate of dye the same in every experiment but varying the secretion rate an inverse relationship between the concentration of the dye in the gastric juice and the secretion rate was found. This interrelationship could be described by

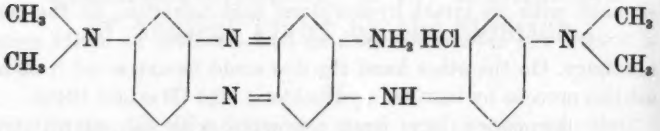
$$C = C_0 - (C_0 - C_\infty) \cdot e^{-k/v} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

where C is the concentration of the dye in the gastric juice at different secretion rates v, C_0 and C_∞ the concentrations in the gastric juice corresponding to zero and infinitely high secretion rates respectively and k a constant. In more recent work from this laboratory NEHM (1958) found an identical relationship when using methylene blue in Heidenhain dogs. It was therefore con-

sidered of much interest to continue also with other dyes to see whether they obeyed the same kind of elimination law. In the present work neutral violet was used. This dye is closely related to neutral red as can be seen from the following formulae.



Neutral red (hydrochloride)
(C. I. no 825)



Neutral violet (hydrochloride)
(C. I. no 826)

Experiments on dogs will be described in which the excretion of neutral violet was related to the secretory state of the stomach. It will be shown that accumulation of neutral violet occurred when it passed the gastric mucosa from blood to gastric juice.

To further elucidate the central problem of to what extent and how this accumulation took place some experiments on isolated stomachs from mice will be described.

Methods.

Dogs provided with Heidenhain pouches and weighing 22 and 15 kg respectively were used. After a control period to insure that no spontaneous secretion was present, the gastric glands were stimulated by histamine dihydrochloride given by continuous intravenous injection with a special syringe device. With this technique a steady state of the gastric secretion rate at a desired level can usually be obtained (ÖBRINK 1948). In the two dogs that were used the steady states were not quite steady as was expected, probably because of obstructions in the outflow. When the secretion rate had reached a "steady state" level, neutral violet was given continuously also by the intravenous route. This injection was continued until a satisfactory steady state of the coloration of the gastric juice had been maintained for a sufficient length of time. The steady state was usually reached within one hour after the beginning of dye injection. It should be pointed out that in all experiments the

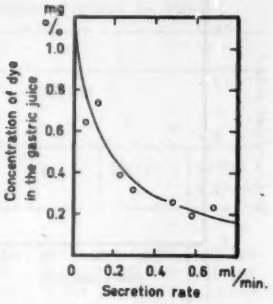


Fig. 1. The relation between the concentration of neutral violet in the gastric juice of a dog and the secretion rate. The solid line is a hyperbolic curve similar to eq. (8).

injection rate of neutral violet was the same, thus giving very similar plasma concentrations of the dye in each experiment provided everything else was kept constant (see discussion).

Neutral violet was prepared by dissolving 1 g in 100 ml H_2O. The solution was filtered and sterilized. The final strength was about 0.87 % (w/v). It was injected at a rate of 2.5 ml/hour which was so slow that the acidity of the dye solution (pH 3.8) was unimportant.

Mice were used for the isolated stomach preparations, which were made exactly according to the method of DAVENPORT (1951) (cf. ÖBRINK 1956). The stomachs were excised and filled with a Krebs-Ringer solution to a pressure of 75 cm H_2O. They were then put in a small beaker containing 5 ml Krebs-Ringer and 5 μg carbacholine-chloride.

The pH of the inside and outside solutions was in several experiments changed from neutral to lower or higher values by admixture of N HCl or N NaOH.

The preparations were incubated for 2 hours at 4.2 atm of oxygen in a water bath at 37° C.

At the start of the experiment both the inner and the outer solutions contained the same concentrations of neutral violet.

Acidity and chloride determinations in the dog experiments were performed in the same way as reported by ÖBRINK (1948). In the isolated stomach experiments pH only was measured. This was done with a glass electrode.

Analysis of neutral violet was performed in a spectrophotometer Beckman model DU or Coleman Jr. As neutral violet changes its color according to pH a series of extinction spectrograms from solutions of similar concentrations were examined at different pH.

In this way two isosbestic points were found at 468 mμ and 568 mμ (cf. ÖBRINK 1948, p. 18). The former represents the color change around pH 6.6 and is useful from alkaline pH values (tested from about pH 9 down to pH 2. The latter represents a color change at an extremely acid pH but is useful up to pH 2. As the extinction in 568 mμ

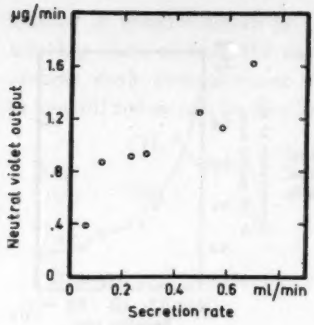


Fig. 2. The relation between the output of neutral violet and the secretion rate of gastric juice. Calculated from Fig. 1.

is much higher than that in 468 mµ the former wavelength was always used in the present investigation. In order to be within a safe pH-range all samples were acidified before being analysed.

In this way extinction values that were unaffected by pH-differences could be obtained.

Attempts to determine neutral violet in plasma have as yet not been successful because the concentrations have been too low.

Results.

The steady state levels from the experiments in one dog are put together in Fig. 1 and Fig. 2. It is no doubt a similar type of appearance for neutral violet as was earlier shown for neutral red by ÖBRINK (1948) and for methylene blue by NEHM (1958). In the other dog a very similar type of appearance curve was also obtained.

The results in mice were collected in Table I.

It can be seen that dye always accumulated on the more acid side, no matter whether this was the mucosal or serosal. It should be noticed, however, that a high percentage of the dye present was taken up by the gastric wall. Most of this uptake was apparently in the glandular part of the stomach, but as yet no histological examinations have been made.

It is evident from Table I that neutral violet accumulated on the more acid side both when the acidity was the result of a secretory activity of the mucosa or obtained by addition of HCl to the Krebs-Ringer solution. Thus an accumulation took place also when acid was added to the inside of a stomach, when the

Table I.

Experiments with neutral violet in mouse stomach in vitro.

Expt. No.	Initially			Finally					
	pH		N. V. conc. mg %	pH		N. V. conc. mg %			
	(i)	(o)	(i) and (o)	(i)	(o)	(i)	(o)	(i)/(o)	in the mucosa[1]
Secreting mucosa									
1	6.82	6.82	0.904	6.46	6.93	0.174	0.115	1.513	14.143
2	6.82	6.82	0.904	3.68	6.84	0.558	0.001	558.000	15.162
3	6.82	6.82	0.904	6.13	6.76	0.188	0.080	2.350	12.113
4	6.82	6.82	0.904	5.85	7.10	0.318	0.100	3.180	21.096
5	6.99	6.99	0.929	6.23	6.99	0.380	0.174	2.183	13.086
6	6.99	6.99	0.929	6.30	7.40	0.375	0.140	2.679	12.025
7	6.99	6.99	0.929	4.25	7.40	0.920	0.108	8.519	10.915
8	6.99	6.99	0.929	6.37	7.23	0.406	0.146	2.780	11.263
13	6.97	6.98	0.961	3.48	7.50	0.590	0.054	10.926	15.279
14	6.97	6.98	0.961	6.08	7.38	0.192	0.052	3.692	20.533
15	6.97	6.98	0.961	4.77	7.38	0.478	0.038	12.579	15.936
16	6.97	6.98	0.961	6.45	7.04	0.138	0.070	1.971	10.200
39	6.88	6.88	0.720	4.84	7.07	0.536	0.148	3.622	8.668
40	6.88	6.88	0.720	4.50	7.18	0.640	0.138	4.638	9.296
42	6.88	6.88	0.720	4.65	7.05	0.572	0.148	3.865	7.574
Secreting mucosa (acid added to (o), alkali to (i))									
9	8.99	3.23	0.774	6.52	5.70	0.182	0.308	0.591	9.617
10	8.99	3.23	0.774	5.99	6.52	0.270	0.176	1.534	11.134
11	8.99	3.23	0.774	6.52	6.18	0.166	0.268	0.619	6.434
12	8.99	3.23	0.774	6.36	6.31	0.270	0.228	1.184	10.184
17	8.99	3.18	0.883	6.69	6.22	0.280	0.332	0.843	7.715
18	8.99	3.18	0.883	7.13	5.58	0.320	0.504	0.635	9.113
19	8.99	3.18	0.883	6.58	6.47	0.360	0.404	0.891	7.769
20	8.99	3.18	0.883	5.50	6.51	0.590	0.340	1.735	10.279
Secreting mucosa (acid added to (i), alkali to (o))									
21	3.82	7.65	0.730	3.13	7.18	0.780	0.192	4.063	7.643
22	3.82	7.65	0.730	3.40	7.52	0.954	0.200	4.770	9.145
23	3.82	7.65	0.730	3.44	7.13	0.656	0.176	3.727	10.338
24	3.82	7.65	0.730	3.49	7.23	0.684	0.208	3.288	8.584
Nonsecreting mucosa (2 : 4-dinitrophenol added to (i) and (o))									
25	3.63	7.15	0.715	5.41	6.67	0.388	0.240	1.617	7.804
26	3.63	7.15	0.715	5.53	6.50	0.364	0.244	1.492	6.703
27	3.63	7.15	0.715	5.45	6.70	0.388	0.234	1.658	6.108
28	6.53	6.53	0.692	6.40	6.42	0.248	0.242	1.025	8.280
29	6.53	6.53	0.692	5.94	6.43	0.272	0.216	1.259	8.886
30	6.53	6.53	0.692	6.29	6.45	0.188	0.232	0.810	10.350
31	6.58	6.58	0.678	6.51	6.52	0.242	0.208	1.163	7.113
32	6.58	6.58	0.678	6.26	6.51	0.216	0.216	1.000	8.377
33	6.58	6.58	0.678	6.18	6.47	0.216	0.242	0.893	9.812
34	6.58	6.58	0.678	6.43	6.48	0.212	0.242	0.876	9.843
35	6.57	6.57	0.736	6.28	6.54	0.192	0.216	0.889	10.768
36	6.57	6.57	0.736	6.26	6.49	0.184	0.200	0.920	9.914
37	6.57	6.57	0.736	6.43	6.50	0.156	0.222	0.703	10.305

[1] calculated as mg dye per 100 g mucosa wet weight.

N. V. = neutral violet; (i) = inside; (o) = outside.

(In expts 25, 26 and 27 acid was added to the inside solution.)

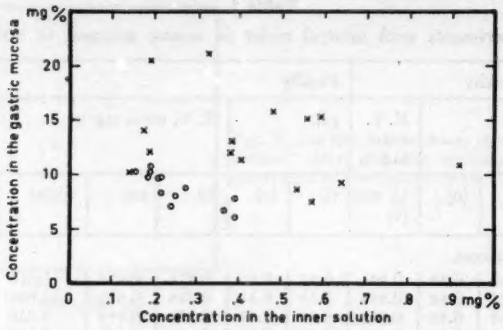


Fig. 3. The relation between the average concentration of neutral violet in the wet gastric mucosa and the final concentration in the solution on the mucosal side (×). In the experiments marked (○) 2:4-dinitrophenol was added to both sides.

secretory activity was abolished by 2 : 4-dinitrophenol (DNP) at a concentration of 1:1,000 (exp. no. 25, 26 and 27). That this dosage of DNP was sufficient to inhibit the secretory activity is evident from exp. 28 through 37. From those experiments it seemed that the accumulation of dye in the gastric mucosa itself was somehow related to the secretory activity. This is probably best seen in Fig. 3 and Fig. 4, where the final concentrations in the mucosa are correlated with the final concentrations in the inner or outer solutions repectively. (In these figures the experiments with acid or alkali added are not included except those with DNP.)

Even if there is very little evidence for a close relationship between the concentration of dye in the stomach wall and its surroundings, it seems reasonably well established that in a stomach with the secretion cut off, the partition of dye, especially between the stomach wall and its serosal fluid, is markedly changed.

Discussion.

It was found in experiments in Heidenhain dogs that the concentration of neutral violet excreted in the gastric juice was much higher than the concentration in the blood. The relation between the concentration in the gastric juice and the gastric secretion rate is seen in Fig. 1 and is similar for neutral red (Öbrink, 1948) and methylene blue (Nehm, 1958). In order to study the

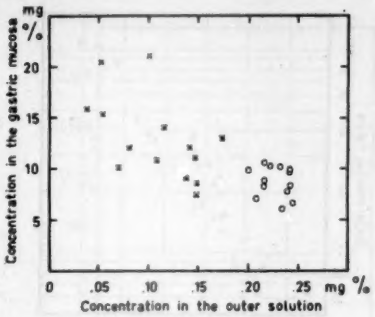


Fig. 4. The relation between the average concentration of neutral violet in the wet gastric mucosa and the final concentration in the solution on the serosal side (×). In the experiments marked (c) 2:4-dinitrophenol was added to both sides.

partition of the dye between gastric juice and plasma more closely, in vitro preparations of stomachs from mice were used. They also showed an accumulation of neutral violet, the dye always being accumulated on the more acid side of the membrane.

Experiments in vitro.

It was shown (Table I) that a great deal of the dye was taken up by the tissue. The present work was not concerned with the question where this uptake took place. It was, however, evident that the uptake was due to some kind of glandular activity as the nonglandular part of the stomach did not show any appreciable coloration. Of the dye remaining in solution most was found where the acidity was the highest. Fig. 5 shows that the greater the difference between the final pH values of the solutions on the two sides, the more was the dye accumulated. This curve does not really show any theoretically deduced interrelationship between partition and pH, and is only intended as a way of representing our findings.

If R is the ratio of dye concentration of the inside solution (i) to the outside (o), R is apparently some function of the acidities of the two sides, thus

$$R = f_1 (H^+_{(i)} / H^+_{(o)}) \quad \ldots\ldots\ldots\

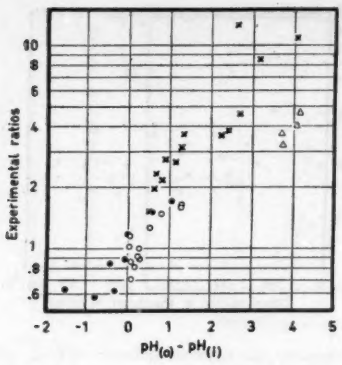


Fig. 5. The experimental ratios of the final neutral violet concentration inside/outside as related to the pH-difference outside-inside.

× = normally secreting mucosa;
• = acid added to (o), alkali to (i);
△ = acid added to (i), alkali to (o);
○ = 2:4 DNP added to both sides.

(△) and where the 2 hours of incubation may have been too short a time for the concentrations to reach equilibrium, the results form a fairly straight line passing through R = 1 for ($pH_{(o)} - pH_{(i)}$) = 0. Thus function (3) could be written.

$$\log R = \text{constant} \times (pH_{(o)} - pH_{(i)}) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (4)$$

and if the constant is put equal to $\frac{1}{n}$

$$R^n = \frac{H^+_{(i)}}{H^+_{(o)}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (5)$$

with the numerical value of n about 3. This function so far is only a mathematical description of the observed events, but gives the fundamental information that the partition of neutral violet between inside and outside is unity for equal pH:s on both sides.

As was shown in the experiments with 2:4-dinitrophenol an accumulation in one of the bathing fluids seemed to occur also after an uncoupling of the energy-rich phosphates, which at least indicates that the accumulation is not necessarily energy dependent but may be a true equilibrium.

One way of explaining the accumulation without the need of having to put energy into the system could be that neutral violet, which is a weak base penetrated the gastric mucosa only in an unionized state as if the mucosa was a lipid membrane. In our experiments we used the conjugated acid (here denoted as VNH_3^+) of the neutral violet base VNH_2 which in water solution gives

$$VNH_3^+ + H_2O \rightleftharpoons VNH_2 + H_3O^+$$

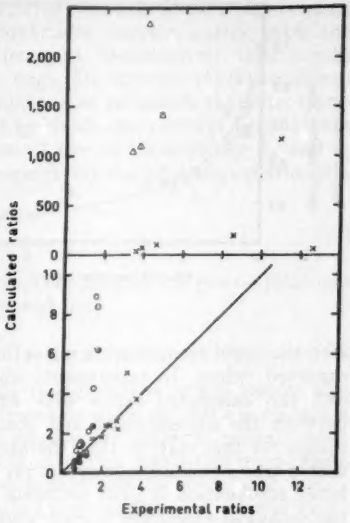


Fig. 6. A comparison between the calculated ratios (according to eq. (6) and the experimental ratios of the final neutral violet concentration inside/outside. (The meaning of ×, ●, △ and ○ the same as in fig. 5.)

The dissociation constant for this reaction (pKa) is 6.6 according to WOISLAWSKI (1953). The amount of free base will obviously depend on the pH of the solution. In the gastric juice the reaction will proceed to the left leaving low activities of the diffusible VNH_2, whereas in a higher pH the opposite will be the case. The equibrilium would be reached when the activities of the unionized molecules of neutral violet (VNH_2) were equal on both sides. In the acid medium this activity would correspond to a much higher total concentration of the dye ($VNH_2 + VNH_3^+$) than in an alkaline medium.

This approach was earlier used by INGRAHAM and VISSCHER (1935), RAY and JUNG (1951) and SHORE, BRODIE and HOGBEN (1957). They also arrived at a similar relationship between the acidities on both sides of the membrane and the ratio (R) between the concentration of dye in the inside and outside solutions. We have adopted the way in which SHORE *et al.* wrote the formula namely

$$R = \frac{1 + 10^{(pKa - pH_i)}}{1 + 10^{(pKa - pH_o)}} \quad \ldots\ldots\ldots\

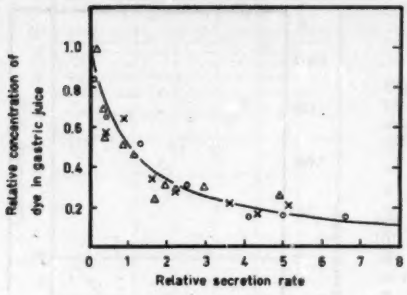


Fig. 7. The relation between the concentration of dye and the secretion rate of gastric juice. Experiments from ÖBRINK (1948) on neutral red (△), from NEHM (1953) on methylene blue (○) and from the present investigation on neutral violet (×) reduced to the same scale. The solid line is a hyperbolic curve similar to eq. (8).

seen the small accumulation ratios fitted very well with the theoretically expected values. In experiments where the pH differences were large and the calculated ratios were expected to be high, discrepancies between the experimental and theoretical values are apparent. The reason for this may be that the above theory is not entirely correct or that a true equilibrium was not yet obtained in the experiments. This latter explanation is quite probable but not so easy to verify, because the technical procedure is such that only one experiment can be performed on each stomach. Doubt may also be expressed on the validity of formula (6). The demand of a perfect impermeability of the gastric membrane towards VNH_3^+ may not be satisfied, but penetration may take place in waterfilled pores, and then the theoretical ratios will never be reached as diffusion in the aqueous phase will counteract the accumulation process.

If the transfer of neutral violet or other basic dyes through the gastric mucosa is not dependent on energy supplying processes it should not be dependent on secretory activity of the gastric glands but only on the presence of hydrochloric acid as the experiments on the isolated mouse stomachs suggested a simple pH partition between the two sides. The findings in the dog experiments, however, seem to show a definite relationship between the secretion rate and the concentration of neutral violet in the gastric juice (see Fig. 1). Since the acidity is low for low secretion rates and increases with increasing volume output one would expect an accumulation that was lower for low secretory rates than for higher ones. The impression is therefore obtained that the results in dogs and isolated mouse stomachs do not agree. The discrepancies may, however, be due to the different experimental procedures rather than to fundamental species differences, but it is not possible to assess this further from the present experiments.

COOKE, DAVENPORT and GOODMAN (1941), in dogs found a slight change in the accumulation ratio of sulfonamides between gastric juice and plasma in relation to the secretion rate. Qualitatively their results resembled those obtained on our dogs. DAVENPORT (1942) explained their results very simply. If the amount of substance that was transported per unit of time is denoted by dx/dt, the relevant permeability coefficient by k and the activities of the sulfonamide by a_p and a_g for the plasma and gastric juice respectively the following equation was obtained:

$$dx/dt = k\,(a_p - a_g) \quad \ldots\ldots\ldots\

juice without the help of a secretory activity, *i. e.* without any change in free energy.

This work was supported by the Swedish Medical Research Council. Miss ANITA WRETE has given valuable technical assistance.

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Addendum.

After this paper was accepted for publication the paper by VARRÓ and JÁVOR (Gastroenterology *35*, 357, 1958) came in our hands. They arrived at conclusions similar to those presented above.

Acta physiol. scand. 1959. 46. 55—61.

From the Department of Clinical Physiology, Karolinska sjukhuset, Stockholm, Sweden.

Oxygen Uptake and Working Capacity in Man During Work on the Bicycle Ergometer With One and Both Legs.

By

HANS DUNÉR.

Received 12 November 1958.

Abstract.

DUNÉR, H. Oxygen uptake and working capacity in man during work on the bicycle ergometer with one and both legs. *Acta physiol. scand.* 1959. *46.* 55—61. — In healthy men the oxygen uptake, working capacity and lactic acid concentration in the blood have been determined during work on the cycle ergometer up to a pulse rate of 170 in steady state with the subject using both legs and each leg separately. The physical working capacity, expressed in kpm/min was with one leg 73—80 % of that using both legs together. The right and left leg gave approximately the same value. The oxygen uptake at pulse rate 170 during work with one leg was 91—100 % of that with both legs. It is presumed that the principal limiting factor in this type of work using large muscle groups is the capacity of the circulation to deliver oxygen to the muscles rather than the amount of muscles taking part in the work.

In performing strenuous physical work using large muscle groups for a period of time of several minutes or longer, various factors can be presumed to determine the physical working capacity.

If the alveolar ventilation is one of these limiting factors the arterial oxygen saturation and oxygen tension should decrease and the carbon dioxide tension should increase during work of this type. In experiments in this laboratory on healthy subjects both in "steady state" work (pulse 170 beats per minute) and during exhaustive work, the arterial oxygen tension decreased somewhat but there was an insignificant decrease of the oxygen saturation (HOLMGREN and LINDERHOLM 1958). Moreover the carbon dioxide tension decreased slightly, indicating that lung ventilation had been more than adequate to eliminate the carbon dioxide.

Another possible limiting factor is the myocardial function. If this is the case then during high work loads the systolic blood pressure should presumably decrease. To the contrary, however, both in the "steady state" work and exhaustive work the systolic and diastolic pressure increases in a linear relationship to pulse rate up to a frequency above 180 beats per minute (HOLMGREN 1956).

Still another of these factors may be the distribution of blood to the working muscles. Thus if the dilatation of the arteries in the working muscles reaches a maximum during work, this should limit the blood flow. The muscle capillary bed and thereby the diffusion surface for gas exchange between blood and tissue may also be a limiting factor. If this is the case during work with large muscle groups involved, the working capacity of a subject should increase linearly with an increase of the muscle mass used, *e. g.* should be doubled if two instead of one leg are used on the bicycle ergometer.

The purpose of this investigation has been to examine the last mentioned problem.

Material and Methods.

The subjects were 5 healthy untrained men, ages 25 to 32. The working capacity test was performed in the sitting position on the bicycle ergometer and the calculation of the working capacity was done according to SJÖSTRAND (1947) and WAHLUND (1948). The test consisted of a series of work loads performed continuously, beginning with 200—300 kpm/min with successive increases of 200—300 kpm/min every 6 min until the pulse rate reached 170. The physical working capacity is defined as that work performed at a pulse frequency of 170 beats per minute in the "steady state" (*i. e.* at a pulse frequency

Table I.

Physical working capacity with both legs and each leg separately.

Case	Physical working capacity kpm/min			
	Right leg	Left leg	Both legs	One leg as % of both
1	750	750	1,000	75
2	550	550	750	73
3	750	700	900	80
4	700	700	900	78
5	1,000	1,000	1,350	74

not varying by more than 10 beats per minute on each work load between the second and the sixth minute of work).

The lactic acid in the blood was determined according to BARKER and SUMMERSON (1941) as modified by STRÖM (1949) and HOLMGREN (1956). Finger puncture samples were collected. To secure arterialized blood the hand was kept in hot water between punctures.

The expired air was collected in Douglas bags. The volume was determined with a "Nordgas" gasometer and oxygen uptake determination was done according to HALDANE and PRIESTLY (1935).

Procedure.

The subjects had a preliminary test to determine their approximate working capacity. An ECG recording was taken both at rest and during this test to rule out abnormalities.

Two days later another test of working capacity was performed and the ECG in a single lead was recorded to determine pulse frequency. The oxygen uptake and the lactic acid in blood was determined at rest and during each work load. After two days interval, the same procedure was repeated using just one leg to perform the work, and then repeated again with the other leg after two more days. The foot was held to the pedal by metal clips. In the single leg test, metal springs (30 cm in length) were attached to the functioning pedal in such a manner as to be stretched when this pedal was on the downstroke in order to simulate the extension phase of work in the one and two legged experiments. The non-working leg rested on a platform arranged somewhat to the side and in front of the subject.

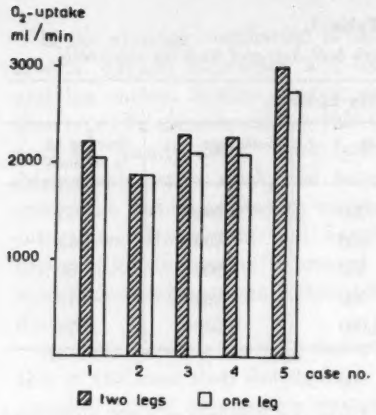

Fig. 1. Oxygen uptake in ml/min during work with both legs together and the mean value of the one legged test at pulse rate 170.

Results.

The physical working capacity as defined above during work with both legs was in the five cases 750—1,350 kpm/min (see Table I). The working capacity with one leg, calculated in the same way, was 550—1,000 kpm/min. The working capacity determined with one leg was thus 73—80 % of that with both legs.

The oxygen uptake at pulse rate 170 during work with both legs was 1,877—2,991 ml per minute (Fig. 1). The corresponding values during work with one leg were 1,876—2,750 ml per minute, *i. e.* 91—100 % of the values with both legs.

Fig. 2 presents a summary of the material and indicates the increase of oxygen uptake in ml per pulse stroke with the increase of work load. There is then an increased peripheral utilization of oxygen with no significant difference of utilization during work with one or both legs.

The amount of the lactic acid in the blood is unchanged during the lower work loads, but then increases rather rapidly, as seen in Fig. 3. The increase in the one leg test tends to occur at lower work loads and is also more pronounced.

Discussion.

The working capacity calculated as the work performed in steady state with a pulse rate of 170 is only 20—27 % lower with one leg than with both legs during the same conditions. However,

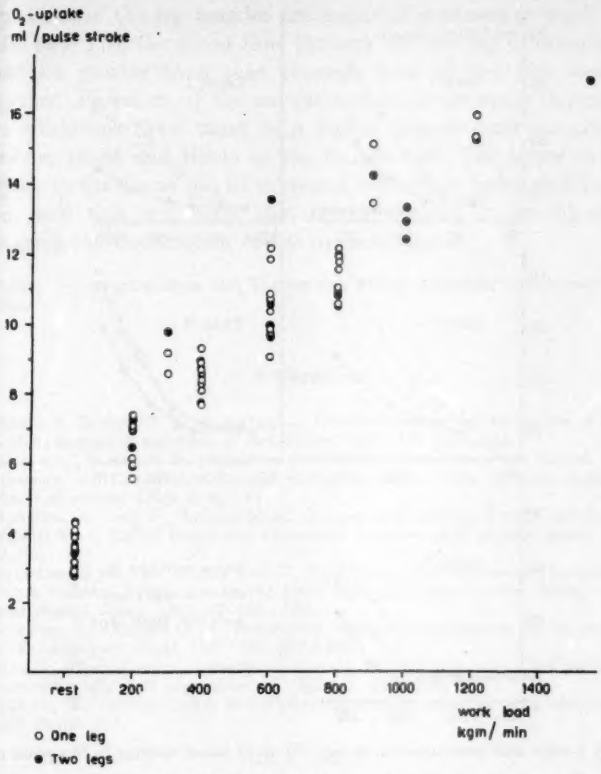

Fig. 2. Oxygen uptake in ml/pulse stroke relative to the increasing work loads in kpm/min.

the mechanical efficiency with one leg is lower than with two legs, so it is not valid to directly compare the work performed under these two circumstances. It is more valid to compare the oxygen uptake during work with both legs and one leg. As seen in Fig. 1, the oxygen uptake in the five cases examined was approximately the same during work with one leg as with both legs. If the size of the diffusion surface in the capillaries between blood and muscles were the limiting factor of the working capacity, the oxygen uptake per pulse beat would be about twice as much with both legs as with one. The results thus seem to show that during steady

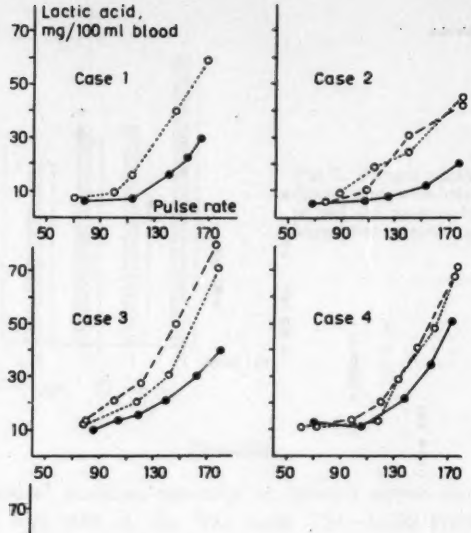

Fig. 3. Lactic acid concentration in mg/100 ml of blood relative to the pulse rate.

state work with two legs, the total working capacity of each leg is not utilized and that the limiting factor is the amount of oxygen distributed by the circulation to the muscles rather than the amount of muscle tissue taking part in the work. This is in accordance with other observations that the oxygen-transporting capacity of the circulation *i. e.* cardiac output and total amount of hemoglobin limit the working capacity during this type of work (KJELLBERG, RUDHE and SJÖSTRAND 1949).

It is possible that the production of lactic acid in the tissue may be of value in the utilization of oxygen in the blood. During work with two legs and with one leg, it is probable that the circulating blood volume is about the same. If one supposes that the

capillaries in the leg muscles are maximally dilated at work with pulse rate 170, the blood flow through the one leg working alone must be greater than that through each of the legs working together. Therefore, if the oxygen uptake is the same during the two situations there must be a higher oxygen gradient existing between blood and tissue in the former case. The lower oxygen tension in the tissue can be supposed to increase lactic acid formation, and this will raise the oxygen tension in the blood by changing the dissociation of the oxyhemoglobin.

Aided by a grant from the Therese and Johan Andersson's memorial foundation.

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Acta physiol. scand. 1959. *46*. 62—87.

From the Pharmacological Institute, University of Uppsala, Sweden.

Relaxation of Glycerol-extracted Muscle Fibre Bundles Induced by Zinc in the Presence of ATP and Other Polyphosphates.

By

K. A. PAUL EDMAN.

Received 24 November 1958.

Abstract.

EDMAN, K. A. PAUL. Relaxation of glycerol-extracted muscle fibre bundles induced by zinc in the presence of ATP and other polyphosphates. *Acta physiol. scand.* 1959. *46*. 62—87. — A survey of factors affecting the relaxing effects of zinc on glycerol-extracted psoas fibre bundles has been made with isometric recording technique. No relaxation is obtained with zinc alone. In the presence of 0.05—0.4 mM adenosinetriphosphate (ATP) and 1 mM magnesium complete relaxation of a contracted fibre bundle is obtained with 0.1—0.4 mM zinc. The relaxing effect decreases at high ATP concentrations, probably due to a decrease in the free zinc ion concentration in the bath caused by complex formation between zinc and ATP. The ATPase activity of the fibre bundles is abolished during relaxation with 0.1 mM zinc + 1 mM magnesium + 0.2 mM ATP. Magnesium and zinc have mutually antagonistic effects on contractility, but the relaxing effect of zinc is probably not due to a simple competition with magnesium. Calcium (10 mM) increases the rate of relaxation induced by zinc + ATP. Relaxation can be induced by 0.2 mM zinc in the presence of 1 mM

magnesium and 0.4 mM inosinetriphosphate(ITP) or uridinetriphosphate instead of ATP. The visco-elastic effects of zinc + ITP are much more pronounced than those of zinc + ATP in equimolar bath concentrations. Zinc does not modify the relaxing effect of inorganic pyrophosphate and tripolyphosphate, nor the effect of adenosinediphosphate.

As demonstrated in an earlier study (EDMAN 1958), it is possible to produce relaxation of a contracted glycerol-extracted muscle fibre bundle under isometric conditions with certain bivalent metal ions in the presence of ATP.[1] The relaxing effect of zinc appears even at low zinc concentrations (0.025 mM) in the presence of 1 mM magnesium and 0.4 mM ATP. The zinc effect is fully reversible; the contraction-relaxation cycle can be repeated numerous times without a noteworthy decrease in the contractility. In a later investigation (EDMAN 1959) the relaxing effect of zinc + ATP was studied under isotonic conditions, and, in addition, the effect of zinc + ATP on the visco-elastic properties of resting fibre bundles was recorded.

The present study is aimed at further investigation of the contraction-inhibiting and relaxing effects of zinc, including among other aspects the influence of magnesium and calcium, ATP and other nucleotides (ITP, UTP, ADP) and inorganic polyphosphates. The effects of zinc on the ATPase activity in the fibre bundles will also be presented, as well as determinations of the effect of zinc + ITP on the visco-elastic behaviour of resting fibre bundles subjected to stretch.

Methods.

I. *Contraction-relaxation experiments.*

Glycerol-extracted fibre bundles from rabbit psoas were used. The technique used in extraction and preparation of these bundles as well as in the isometric recording of the tension development has been described (EDMAN 1957). The length of the fibre bundles was 10.0 mm; the cross section was 65—150 × 145—285 μ. The fibre bundles were given an initial tension of 17.2 mg in the experiments where only contraction was studied. In the relaxation experiments the initial tension was 22.2 mg.

[1] The following abbreviations will be used: ADP, adenosinediphosphate; ATP, adenosinetriphosphate; CTP, cytidinetriphosphate; EDTA, ethylenediamine tetraacetate; GTP, guanosinetriphosphate; ITP, inosinetriphosphate; PP, inorganic pyrophosphate; TPP, inorganic tripolyphosphate; UTP, uridinetriphosphate.

A potassium chloride solution (pH 7.30), containing 10 mM Veronal (diethylbarbituric acid), 100 mM potassium and 1 mM magnesium, was used throughout as medium after the glycerol-extraction. Hereafter, this solution will be called "veronal buffer". The magnesium concentration will not be specially mentioned in the rest of the communication unless the concentration was different from 1 mM.

All nucleotides used were obtained as "chromatographically pure" substances: ATP, dibarium salt, Pabst, lot. no. 129 (for analytical data, see EDMAN 1958); ATP, disodium salt, Pabst, lot. no. 109 A (only used in the visco-elasticity measurements and in the determinations of the ATPase activity; for analytical data, see EDMAN 1959); ADP, monosodium salt, Sigma, lot. no. 18—671; ITP, trisodium salt, Pabst, lot. no. 2601; UTP, trisodium salt, Pabst, lot. no. 706. $Na_4P_2O_7 \cdot 10\ H_2O$ and $Na_5P_3O_{10} \cdot 6\ H_2O$, pro analysi, were provided by the courtesy of Chemische Werke Albert, Wiesbaden-Biebrich. For data on the quality of the other chemicals used, see EDMAN (1958). The water used for washing and for preparation of solutions was double distilled in borosilicate glass. The pH determinations were performed with a glass electrode. The experiments were carried out at room temperature (20—22° C).

If not otherwise stated, the concentrations given refer to *total* concentration in the bath solution. In calculating the concentrations of free zinc, magnesium and ATP the same stability constants (log K) were used as earlier (EDMAN 1959), viz. 4.00 for the MgATP complex and 4.76 for the ZnATP complex.[1]

Fisher's t-test was used in determining the statistical significance.

II. *Determination of the ATPase activity in the glycerol-extracted muscle.*

The aforementioned veronal buffer was used as medium. About 3 g glycerol-extracted psoas musculature was first reduced to a pulp in a porcelain mortar with about 5 ml buffer solution. After this coarse pretreatment the muscle tissue was homogenized manually in a Potter-Elvehjem homogenizer with a glass pestle. The homogenization took place at + 4° C during 5-min periods with 10-min intervals. After 8 to 10 such treatments the muscle mass consisted of an undifferentiated brei without observable fibre structures on microscopic inspection (× 120). After being washed three times with 40 ml buffer solution the muscle mass was suspended in 15 ml 50 % water solution of glycerol containing 10 mM sodium phosphate (pH 7.0), *i. e.* the same solution as used for the initial glycerol-extraction of the muscle bundles (EDMAN 1957). The muscle suspension was subsequently stored at — 20° C. Before use 1 ml muscle suspension was washed three times with 20 ml veronal buffer by means of gentle shaking for 5 min followed by 15 to 20 min centrifugation (3,500 rpm). Finally, the suspension was diluted

[1] The stability constants obtained by Prof. G. WEITZEL (pers. comm.) have recently been published (WEITZEL and SPEHR 1958).

with veronal buffer to 40 ml, and the muscle tissue was further homogenized in this volume by a few minutes' treatment in the homogenizer. Washing and centrifugation took place at + 4° C.

The ATP solution for test and control experiments was prepared from the same stock solution of Na_2H_2ATP adjusted to pH 7.30 with KOH; the veronal buffer (pH 7.30) was used as solvent. The ATP solution for the tests also included zinc. Each incubation experiment was made up of 2 ml muscle suspension (~ 0.3 mg N), 6 ml veronal buffer and 4 ml ATP solution. The incubation was carried out at + 25° C during slight continuous shaking. The reaction was stopped after 5 min by addition of 4 ml trichloracetic acid to a final concentration of 2.5 %. The mixture was then centrifuged for 15 min (3,500 rpm) at + 4° C.

Two aliquots of 1.5 ml of the supernatant fluid were taken for determination of inorganic phosphate. The method of MARTIN and DOTY (1949) was used, modified to permit use of one-tenth the volume of reagents with micropipettes. The determination was carried out in cuvettes with 1 cm light path in a Beckman model B spectrophotometer.

Because of the spontaneous hydrolysis of ATP, parallel experiments in which trichloracetic acid was added to the muscle suspension before the addition of ATP were carried out both in the presence and in the absence of zinc. The phosphate value in these experiments constituted the zero level in the determination of the ATPase activity.

For determination of the dry weight 10 ml of the final muscle suspension was used. Desiccation took place at + 105° C. The nitrogen content was determined with micro-Kjeldahl technique.

III. *Visco-elasticity measurements.*

The visco-elasticity determinations with zinc + ITP were carried out in the manner described for 0.2 mM zinc + 0.4 mM ATP in an earlier study (EDMAN 1959). The bundles, given an initial tension of 22.2 mg, were first contracted isometrically with 0.4 mM ATP for 2 min and were thereafter washed with veronal buffer alone for 2 min and treated with 0.2 mM zinc for an additional 5 min. Isometric relaxation was then induced with 0.2 mM zinc + 0.4 mM ITP and the tension was recorded for 5 min, when constant tension had certainly been reached. After having been immersed in the solution of 0.2 mM zinc + 0.4 mM ITP for 60 min, the fibre bundle was stretched by 0.83 % of its length. The stretching was achieved in 2 sec. The tension was recorded for 5 min, when a constant level had been reached. The control experiments in the visco-elasticity determinations with zinc + ATP (EDMAN 1959) were carried out on the same fibre bundle preparation as used in the present study with zinc + ITP and have therefore been used as controls in the experiments with zinc + ITP also.

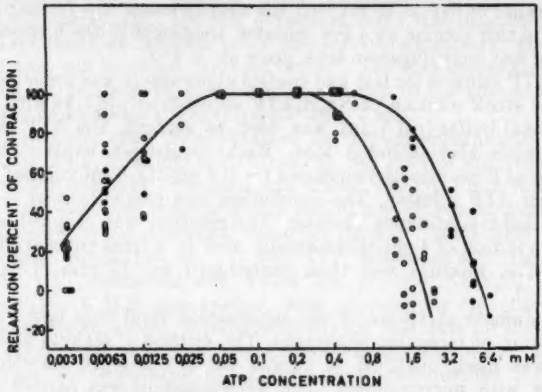

Fig. 1. Relaxing effect of 0.1 mM (open circles) and 0.4 mM (filled circles) zinc in the presence of different concentrations of ATP. Fibre bundles contracted with 0.4 mM ATP before start of relaxation (see this page for details).

Ordinate: relaxation, drop in tension, expressed in percentage of previous contraction.

Abscissa: ATP concentration during relaxation. Curves fitted by sight.

Results.

1. Relaxing effect at varied concentrations of zinc and ATP.

Relaxation induced by 0.1 mM and 0.4 mM zinc at varied ATP concentrations.

Fig. 1 illustrates the relaxing effect of zinc in two different concentrations (0.1 mM and 0.4 mM) with variation of the ATP concentration over a wide range (0.0031 to 6.4 mM). The fibre bundles were first contracted by 0.4 mM ATP for about 2 min. When the ATP concentration during relaxation exceeded 0.4 mM, the zinc + ATP combination was applied to the fibre bundle in direct connection with the contraction. As shown earlier (EDMAN 1958), the tension in a contracted fibre bundle is decreased by the mere removal of the ATP by washing. Therefore, in order to determine the relaxation with zinc at ATP concentrations below 0.4 mM the fibre bundle was first immersed in buffer solution containing the lower ATP concentration for 10 to 15 min after the contraction before zinc + ATP was applied; in some experiments the bundles were treated instead with ATP-free buffer

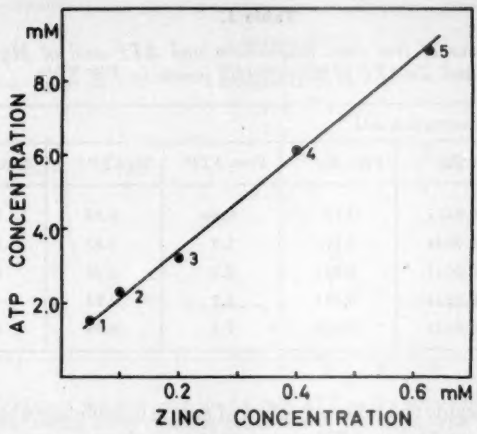

Fig. 2. Relation between zinc concentration (abscissa) and lowest ATP concentration (ordinate) just nullifying the relaxing effect of zinc in fibre bundles previously contracted by 0.4 mM ATP alone. The values represent the mean of four experiments. Concentrations (mM) of free Zn, Mg, ATP and the complexes of ZnATP and MgATP existing at the points numbered 1—5 are given in Table I.

solution after the contraction. With the addition of zinc + high concentrations of ATP, the tension initially increases beyond that obtained with 0.4 mM ATP alone and thereafter decreases to an equilibrium level (for interpretation, see later). The tension was always recorded until equilibrium had definitely been reached.

As shown by Fig. 1, complete relaxation was obtained with both 0.1 mM and 0.4 mM zinc in the presence of ATP in concentrations between 0.05 mM and 0.2—0.4 mM. (Complete relaxation signifies return to initial tension.)

The relaxing effect decreases with decreasing ATP concentration; no relaxation at all is obtained in the total absence of ATP. An ATP concentration as low as 0.006 mM is sufficient, however, to give a distinct relaxation with 0.1 and 0.4 mM zinc. At these ATP concentrations, suboptimal in respect to relaxation, the effects of 0.1 mM zinc and 0.4 mM zinc do not differ significantly from each other (left side of Fig. 1).

As shown in the right side of Fig. 1, the relaxing effect of zinc also decreases at high concentrations of ATP. If the ATP concentration is increased sufficiently while the zinc concentration

Table I.

Concentrations of free zinc, magnesium and ATP and of MgATP and ZnATP at the different points in Fig. 2.

Number of point in Fig. 2.	Concentration mM				
	Free Zn^{++}	Free Mg^{++}	Free ATP	MgATP	ZnATP
1	*0.0014*	0.14	0.60	*0.86*	0.049
2	*0.0014*	0.073	1.3	*0.93*	0.099
3	*0.0017*	0.047	2.0	*0.95*	0.20
4	*0.0015*	0.021	4.7	*0.98*	0.40
5	*0.0015*	0.014	7.2	*0.99*	0.62

remains constant, the relaxing effect of zinc is completely abolished, and, with even higher ATP concentrations, the tension increases beyond that obtained with 0.4 mM ATP alone. The higher the zinc concentration the higher the ATP concentration must be for the relaxing effect to begin to decrease. This is illustrated in Fig. 1 for zinc concentrations of 0.1 mM and 0.4 mM.

Mechanism underlying the decrease in relaxing effect of zinc at high ATP concentrations.

Fig. 2 shows the relation between the zinc concentration and the lowest ATP concentration that proved able to completely abolish the relaxing effect of zinc in fibre bundles previously contracted by 0.4 mM ATP. (The zinc concentrations tested, 0.05—0.62 mM, give complete relaxation in the presence of 0.4 mM ATP.) As may be seen, there is a direct proportionality between the total concentration of zinc and the total concentration of ATP necessary to prevent relaxation. Table I presents the concentrations of free zinc, magnesium and ATP as well as the concentrations of the MgATP and ZnATP complexes existing at the different points in Fig. 2. As is seen, the concentration of free zinc ions is approximately constant, 0.0015 mM. The MgATP concentration is also roughly constant (0.86—0.99 mM); almost all magnesium occurs as MgATP complex.

What makes the tension the same at the different points in Fig. 2? The tension cannot be determined solely by the concentration of MgATP since complete relaxation is obtained at

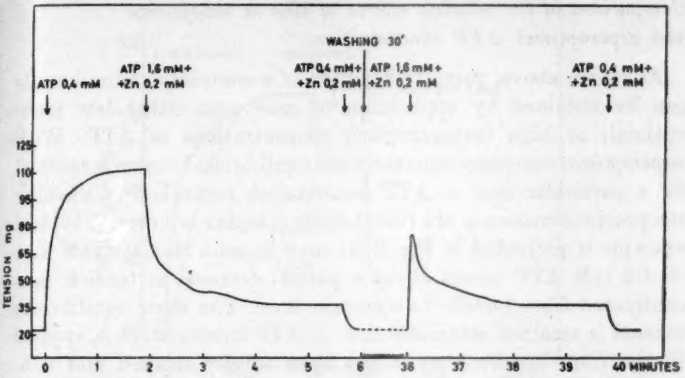

Fig. 3. Effect of 0.2 mM zinc + 1.6 mM ATP on the same fibre bundle in contracted and resting states.[1] Washing before second treatment with 0.2 mM zinc + 1.6 mM ATP was performed with veronal buffer solution alone. Note that the same equilibrium tension is obtained irrespective of the previous tension of the fibre bundle. Cross section of the fibre bundle: 84 × 178 μ.

0.95 mM MgATP when 0.017 mM free zinc is present, as demonstrated on page 71. That the tension does not drop instantaneously after the addition of the zinc + ATP mixture but initially increases and thereafter decreases to an equilibrium level (see earlier) indicates that the relaxing effect of zinc is dependent upon an accumulation of zinc in the fibre protein. This in turn will be determined by the free zinc ion concentration in the medium. Therefore, it seems probable that the relaxation-inhibiting effect of ATP in high concentrations is due to the decreased concentration of free zinc ions. With an adequate increase in the ATP concentration the zinc ion concentration in the bath solution drops to such an extent that the accumulation of zinc in the fibre protein becomes insufficient for relaxation. Since the free zinc ion concentration is constant at the various points in Fig. 2, it is conceivable that the inhibition of contractility is also the same at these points. That constant tension is obtained in spite of differences in the concentration of free ATP is probably due to the fact that ATP is optimal for contraction within the concentration range studied (cf. Fig. 6, EDMAN 1957 and Table 8, WEBER and PORTZEHL 1954). The 0.0015 mM concentration of free zinc ions is accordingly just below the threshold with respect to relaxation under the conditions used in these experiments.

[1] At the arrows in this and the other figures the previous bath solution was completely exchanged for the new one.

Comparison of the relaxing effects of zinc at suboptimal and superoptimal ATP concentrations.

As shown above, partial relaxation of a contracted fibre bundle can be obtained by application of zinc with either low (suboptimal) or high (superoptimal) concentrations of ATP. With *superoptimal* concentrations the same equilibrium tension is reached for a particular zinc + ATP combination regardless of whether the previous tension in the fibre bundle is higher or lower. A typical example is presented in Fig. 3. It may be seen that 0.2 mM zinc + 1.6 mM ATP brings about a partial decrease in tension in a contracted fibre bundle to a certain level. The same equilibrium tension is attained when the zinc + ATP combination is applied to the fibre bundle after it has been totally relaxed and subsequently washed with zinc-free buffer solution for 30 min. The initial, rapid increase in tension that takes place when zinc + ATP are applied to the relaxed and washed fibre bundle probably depends upon the slowness of the accumulation of zinc in the fibre bundle. These observations show that the changes induced by zinc + ATP in the contractile system are fully reversible. This agrees with the good repeatability of a contraction-relaxation cycle induced with zinc + ATP, as demonstrated earlier (EDMAN 1958).

With *suboptimal* ATP concentrations, zinc in the presence of ATP is able to decrease the tension in the fibre bundle to a certain level only, but it is not able, on the other hand, to increase the tension to the same level by contracting a fibre bundle that has lower tension previously. This is not surprising since the ATP concentration in the suboptimal range is too low to induce contraction even in the absence of zinc. The threshold concentration of ATP for induction of isometric contraction of glycerol-extracted fibre bundles is approximately 0.02 mM in a zinc-free medium containing 1 mM magnesium.

Relaxation induced by 2 mM zinc in the presence of 5 mM ATP.

In accordance with the foregoing observations, it is necessary to increase the total concentration of zinc in the bath solution to a corresponding degree if relaxation is to be obtained with zinc at high ATP concentration. When the total concentration of ATP is 5 mM, as is needed for relaxation with the Marsh-

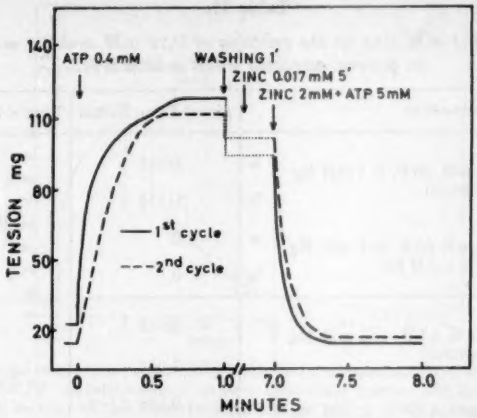

Fig. 4. Two contraction-relaxation cycles produced in the same fibre bundle with 2 mM zinc + 5 mM ATP. Washing at height of contraction performed with veronal buffer solution only. After the first relaxation the fibre bundle was washed with veronal buffer solution for 30 min before the second contraction was started. Cross section of the fibre bundle: 103 × 182 μ.

Bendall factor for instance (HASSELBACH and WEBER 1953, WEBER and PORTZEHL 1954), it is necessary to have about 2 mM total concentration of zinc for complete relaxation in the presence of 1 mM magnesium. The concentration of 2 mM zinc was found empirically and is close to the lower limit giving complete isometric relaxation. With the combination 2 mM zinc (+ 1 mM magnesium) + 5 mM ATP the free zinc ion concentration is 0.017 mM, the concentration of free ATP 2.1 mM, and that of MgATP 0.95 mM. A typical example of the isometric relaxation with this zinc + ATP combination is given in Fig. 4. The fibre bundle was pretreated 5 min with 0.017 mM zinc so the zinc had time to accumulate to an effective concentration before addition of ATP. (When zinc + ATP is applied without pretreatment with zinc, the fibre bundle responds with an initial contraction before the relaxation starts.) The time required to attain 50 per cent of total relaxation is 4.2 $\pm$ 0.7 sec (mean time from 6 relaxation experiments). The contraction-relaxation cycle is fully repeatable after 30 min washing with buffer solution in the manner described earlier for 0.025 — 0.88 mM zinc + 0.4 mM ATP (EDMAN 1958).

Table II.

Effect of 0.1 mM zinc on the splitting of 0.22 mM and 2.6 mM ATP in glycerol-extracted psoas muscle brei.

Series of experiments			μmole P/mg N/min	Test × 100/control
I	0.22 mM ATP + 1 mM Mg (control)	a	0.547	0
		b	0.559	
	0.22 mM ATP + 1 mM Mg + 0.1 mM Zn	a	0	
		b	0	
II	2.6 mM ATP + 1 mM Mg (control)	c	0.705	73.0
		d	1.036	
	2.6 mM ATP + 1 mM Mg + 0.1 mM Zn	c	0.489	
		d	0.793	

Corresponding tests and controls are indicated by the same letters.

2. *Determination of the ATPase activity.*

A prerequisite for induction of complete isometric relaxation with lower ATP concentrations than that used for the previous contraction of the fibre bundle is inhibition of the ATPase activity. Otherwise ATP would not be able to penetrate sufficiently deeply into the fibre bundle (EDMAN 1957) and would not have a possibility of relaxing the deeper layers that were contracted at a higher ATP concentration.

Table II presents the results of experiments that were carried out for determination of the ATPase activity under conditions corresponding to the contraction-relaxation experiments. The muscle pulp was thoroughly homogenized (see Methods) to allow complete penetration of ATP.

As may be seen from Table II, the ATPase activity is completely abolished with the 0.1 mM zinc + 0.22 mM ATP combination, which gives full relaxation (Fig. 1). On the other hand, with 0.1 mM zinc + 2.6 mM ATP, where the ATP concentration is clearly superoptimal for relaxation (see Fig. 1 and 2) there is only a partial reduction of the ATPase activity as compared with 2.6 mM ATP alone. The decrease in inhibition of the ATPase activity at the high ATP concentration is probably due to the decrease in free

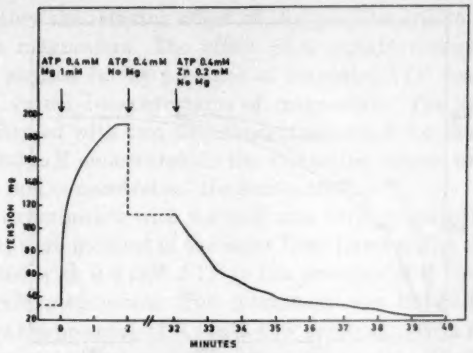

Fig. 5. Partial relaxation produced by removal of magnesium from the medium at unchanged ATP concentration. Complete relaxation follows addition of 0.2 mM zinc. Cross section of the fibre bundle: 150 × 220 μ. Two contraction-relaxation cycles preceded the cycle illustrated.

zinc ion concentration in the bath caused by complex formation between zinc and ATP as described above.

Effects of zinc on the ATPase activity of contractile proteins in solution have been reported and are in agreement with the results on glycerol-extracted muscle demonstrated in this work. Thus, according to MARUYAMA (1958), 1 mM zinc completely abolishes the ATPase activity of crayfish myosin B at + 30° C in the presence of 1 mM ATP, 10 mM calcium and 600 mM potassium. GILMOUR and GRIFFITH (1958) report that zinc in concentrations below 0.015 mM stimulates slightly, while in concentrations above 0.015 mM zinc inhibits the ATPase activity of rabbit myosin at + 25° C in the presence of 1.13 mM ATP, 10 mM calcium and 100 mM potassium; at 0° C even the low zinc concentrations are inhibiting. DICKENS and GLOCK (1951) demonstrated a slight inhibition of the ATPase activity of rat myosin by 0.02 mM zinc at + 37° C in the presence of 3.2 mM ATP, 5 mM calcium and 425 mM potassium.

3. *Significance of magnesium in the relaxation induced by zinc and ATP.*

The magnesium ion stimulates the ATP-induced contraction of glycerol-extracted fibres with optimal effect at 1—10 mM concentration. Reduction of the magnesium in a contracted fibre

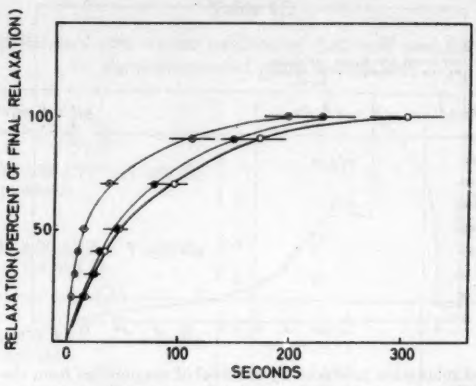

Fig. 6. Effect of magnesium on the rate of the zinc + ATP- induced relaxation of glycerol-extracted fibre bundles.

Ordinate: relaxation expressed in percentage of total drop in tension.

Abscissa: time in sec from start of relaxation.

Contraction induced by 0.4 mM ATP, relaxation by 0.2 mM zinc + 0.4 mM ATP in presence of, respectively, 0.1 mM (⊙), 1 mM (○) and 10 mM (●) magnesium in the medium during contraction and relaxation.

Number of experiments: 5. The standard error of the mean indicated with a horizontal bar. The p-value for the difference between the 0.1 mM and 1 mM magnesium curves at 70 % relaxation is < 0.01. The mean tension in excess of the initial tension in mg/100 μ fibre bundle circumference before and after relaxation was:

before	after
15.0 ± 1.1	1.2 ± 0.6 at 0.1 mM magnesium
25.5 ± 2.0	0.5 ± 0.5 at 1 mM magnesium
24.1 ± 1.8	0.9 ± 0.7 at 10 mM magnesium

bundle by washing lowers the tension appreciably despite the presence of ATP in unchanged concentration (0.4 mM). This is illustrated in Fig. 5. The washing was carried out in magnesium-free veronal buffer for 30 min. Total relaxation was obtained by 0.2 mM zinc + 0.4 mM ATP.

The following experiments were performed in order to investigate whether there is any antagonism between zinc and magnesium,

and, whether the relaxing effect of zinc may be due to a competition with magnesium. The effect of a certain concentration of zinc was studied in the presence of constant ATP concentration but with varied concentrations of magnesium. The experiments were performed with two zinc concentrations, 0.2 mM and 0.0076 mM. At 0.2 mM concentration the relaxation course was studied, at 0.0076 mM concentration the contraction.

In the experiments with 0.2 mM zinc three contraction-relaxation cycles were induced in the same fibre bundle. The contraction was induced with 0.4 mM ATP in the presence of 0.1 mM, 1 mM and 10 mM magnesium. The relaxation was induced with 0.2 mM zinc in the presence of 0.4 mM ATP and magnesium in the same concentration as during the previous contraction. Between each relaxation and the succeeding contraction the fibre bundle was washed for 30 min in veronal buffer containing 0.1 mM magnesium. The order of the different contraction-relaxation cycles was varied. The results obtained with five fibre bundles are presented in Fig. 6. The absolute tension at the beginning and end of relaxation is given in the figure text. The relaxation is expressed as fraction of maximum relaxation. By this means the slope of the curves, which represents the rate of relaxation, becomes independent of the absolute tension. As pointed out earlier (page 69) zinc must probably accumulate to a certain concentration in the fibre protein before the contractility becomes inhibited. In fibre bundles, — like those used in these experiments —, not pretreated with zinc the rate of relaxation is probably determined by the rate of accumulation of zinc in the protein (Fig. 3, EDMAN 1958); the relaxation rate is thus an expression of the time necessary to reach an effective concentration of zinc in the fibre protein. If a simple competitive antagonism exists between zinc and magnesium, an increase in the magnesium concentration would be expected to increase the need for accumulation of zinc in the bundle, and, consequently, cause a slower relaxation.

As is evident from the results in Fig. 6, the contracted fibre bundles are relaxed almost completely to the initial level by 0.2 mM zinc + 0.4 mM ATP at all three magnesium concentrations studied, i. e. not even the highest magnesium concentration used (10 mM) is sufficient to inhibit the final zinc effect. The relaxation takes place more slowly in the presence of 1 mM and 10 mM magnesium than with 0.1 mM magnesium. On the other hand, there is no significant difference in rate of relaxation between 1

Fig. 7. Effect of 0.0076 mM zinc on the isometric contraction of glycerol-extracted fibre bundles induced by 0.24 mM ATP at different concentrations of magnesium.

Ordinate: tension in mg/100 μ fibre bundle circumference.

Abscissa: magnesium concentration (mM).

Filled circles: 0.0076 mM zinc; open circles: control experiments without zinc. Each symbol represents one experiment.

mM and 10 mM magnesium. However, if the relaxing effect of zinc were solely due to a competition with magnesium, the increase in magnesium from 1 mM to 10 mM would be expected further to inhibit the relaxation rate; in fact, this further inhibition would be expected to be much greater than that obtained with the comparatively small increase in magnesium from 0.1 mM to 1 mM. Hence, it seems improbable from these results that the relaxing effect of zinc is due to a simple competition with magnesium.

Fig. 7 shows the tension developed in a series of isometric contraction experiments carried out with 0.0076 mM zinc[1] and 0.24 mM ATP in the presence of magnesium in concentrations varied from 0.01 mM to 10 mM. The zinc concentration was selected so that almost complete inhibition of the contraction was obtained in the presence of the lowest magnesium concentra-

[1] The veronal buffer solution used as solvent in these experiments contained 0.14 μmole zinc/l according to an analysis performed by the Analytical Department of the University of Uppsala.

tion. It is evident that the inhibition of contraction by zinc decreases with increasing magnesium concentration. In the presence of 1 mM and 10 mM magnesium, 0.0076 mM zinc causes no definite inhibition of contraction.

In a quantitative evaluation of the effects of zinc at the different magnesium concentrations demonstrated in Fig. 7, the following complicating factors must be considered:

1. Inhibition of the ATPase activity increases the depth of penetration of ATP which more or less compensates for the inhibition of the contractility. According to GESKE, ULBRECHT and WEBER (1957), the ATPase activity of suspended glycerol-extracted myofibrils is maximal at 1 mM magnesium (pH 7.0, $\mu = 0.1$, ATP 0.4 mM). It is therefore conceivable that the contraction-inhibiting effect of zinc at 0.01 mM magnesium may be underestimated because of deeper penetration of ATP. 2. With an increase in the magnesium concentration, a greater portion of the zinc will appear as free ions in the medium due to displacement of zinc from ATP by magnesium. Therefore, at high magnesium concentrations the contraction-inhibiting effect of zinc might be overestimated, since the effect is probably dependent on the free zinc ion concentration (see page 69).

4. The minimum zinc concentration producing total relaxation.

The zinc-induced relaxation is influenced by both the ATP concentration and the magnesium concentration in the bath. The following experiments were performed to determine the minimum free zinc ion concentration necessary for producing complete relaxation of a contracted fibre bundle in the absence of magnesium in the medium. The fibre bundle was first contracted with 0.4 mM ATP in the presence of 1 mM magnesium. After the contraction the bundle was washed with magnesium-free buffer solution for 10 min and was then immersed in zinc solution (without magnesium) for 30 min after which the solution was exchanged for another with the same composition but, in addition, containing ATP. A series of decreasing zinc concentrations were tested, and for each zinc concentration the optimal concentration of ATP for obtaining relaxation was determined. The lowest total zinc concentration at which complete relaxation could be established in the absence of magnesium was 0.0125 mM, if ATP was present in 0.04—0.08 mM concentration. This corresponds to a free zinc ion concentration of only 0.0025 mM in the medium.

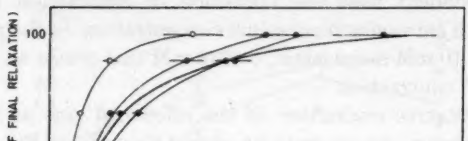

Fig. 8. Effect of calcium on the rate of the zinc + ATP- induced relaxation of glycerol-extracted fibre bundles.

Ordinate: relaxation expressed in percentage of total drop in tension.

Abscissa: time in sec from start of relaxation.

Magnesium (1 mM) consistently present. Contraction induced by 0.4 mM ATP in absence of calcium. Relaxation induced by 0.2 mM zinc + 0.4 mM ATP ± calcium:

(No calcium: ●;
0.1 mM calcium: ⊗;
1 mM calcium: ⊙;
10 mM calcium: ○).

Number of experiments: 4. The standard error of the mean is indicated with a horizontal bar. The p-value for the difference between the control curve (without calcium) and the 10 mM calcium curve is < 0.01 at 70 % relaxation. The tension dropped to the initial tension during relaxation in all experiments.

5. *Influence of calcium on the relaxation induced by zinc and ATP.*

Since some of the relaxing factors described earlier, the Marsh-Bendall factor (Marsh 1952, Bendall 1953, 1954, 1958 a) and EDTA (Bozler 1954, Watanabe 1955, Watanabe and Sleator 1957, Bendall 1958 b) are inhibited by even low concentrations of calcium, it is of interest to investigate whether calcium also inhibits the relaxation induced by zinc + ATP.

Magnesium in 1 mM concentration was consistently present. The fibre bundle was first contracted by 0.4 mM ATP in the absence of calcium for 2 min, and thereafter relaxed by 0.2 mM zinc + 0.4 mM ATP in the presence of 0.1 mM, 1 mM and 10 mM

calcium. Control experiments were performed with no calcium present in the bath during relaxation. Four contraction-relaxation cycles were carried out with the same fibre bundle, *i. e.* one cycle for each calcium concentration and one control cycle. Between each cycle the fibre bundle was washed with veronal buffer alone for 30 min. The order of the experiments in the series was varied. Fig. 8 summarizes the results on the rate of relaxation obtained with 4 fibre bundles. In all cases the tension returned to the initial level during relaxation regardless of the calcium concentration. Accordingly, at the concentrations studied calcium does not notably change the magnitude of the relaxation induced by zinc + ATP. On the other hand, the rate of the relaxation increases in the presence of 10 mM calcium, as shown in Fig. 8.

The effect of 10 mM calcium on the relaxation with zinc + ATP is entirely in accordance with the observation that calcium in that concentration itself induces a partial relaxation. As shown in an earlier study (EDMAN 1958), the relaxation obtained with 10 mM calcium is even greater in the absence of ATP than in the presence of 0.4 mM ATP. These effects of calcium on the zinc + ATP-induced relaxation must further mean that the mechanism acting in the relaxation with zinc is other than in the relaxation with the Marsh-Bendall factor and EDTA.

6. *Relaxation by ADP and inorganic polyphosphates in the presence and absence of zinc.*

It is known that ADP, inorganic pyrophosphate (PP) and tripolyphosphate (TPP) can induce relaxation of glycerol-extracted fibres that have been contracted with ATP (PORTZEHL 1952, WEBER and PORTZEHL 1952, 1954). It is therefore of interest to determine if the relaxation by these phosphate compounds is affected by the presence of zinc.

The procedure in the experiments was as follows. The bundles were first contracted by 0.4 mM ATP for about 2 min. The ATP was then exchanged for ADP, PP or TPP, either directly or after washing for 2 to 15 min with ATP-free buffer solution. When the fiber bundle had relaxed to equilibrium (4—6 min), the bath solution was exchanged for another containing, in addition to ADP, PP or TPP, 0.2 mM zinc. In some experiments two contraction-relaxation cycles were carried out instead on the same fibre bundle (with 10 min washing after the first relaxation), with the

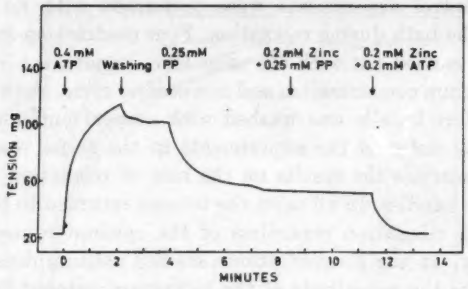

Fig. 9. Relaxing effect of 0.25 mM PP on glycerol-extracted fibre bundles in the absence and presence of 0.2 mM zinc. Washing after contraction performed with veronal buffer solution only. Note that zinc does not cause any decrease in tension in addition to that obtained with PP alone. Complete relaxation is obtained with 0.2 mM zinc + 0.2 mM ATP. Cross section of the fibre bundle: 117 × 168 μ.

relaxation induced in the first cycle in the absence of zinc and in the second cycle in the presence of zinc.

TPP in 0.5 mM concentration (2 exp.) and PP in 1 mM concentration (4 exp.) induces an almost total return of the tension to the initial level. It was possible with even as low as 0.25 mM concentrations of TPP (5 exp.) and PP (3 exp.) to demonstrate a partial relaxation in excess of the tension decrease of about 30 to 50 per cent that takes place as a result of removal of the ATP by washing in buffer solution for 2 to 15 min. ADP in concentrations of 0.25 mM (4 exp.), 1 mM (7 exp.) and 10 mM (3 exp.) produced no demonstrable relaxation in excess of that resulting from washing.[1]

The presence of 0.2 mM zinc does not change the reaction of the fibre bundle to ADP, PP and TPP in the concentrations studied. (The numbers of experiments were the same as stated for the experiments with the various concentrations of ADP, PP and TPP in the absence of zinc.) For purposes of comparison relaxation was finally induced with 0.2—0.4 mM *ATP* + 0.2 mM zinc in all experiments with ADP and in the experiments with 0.25 mM TPP and PP. The tension then dropped to the initial level. A typical example of the relaxation with 0.25 mM PP in the absence and presence of 0.2 mM zinc is given in Fig. 9.

[1] The preliminary finding of a relaxing effect of ADP mentioned in an earlier paper (EDMAN 1957) could not be verified.

Table III.

Comparison of the contraction-inducing effects of ATP, UTP and ITP on glycerol-extracted muscle fibre bundles in the presence of 1 mM magnesium.

Substance	Latency from immersion to start of contraction in sec	Isometric tension 3 min after the start in mg per 100 μ of fibre bundle circumference	Number of expts.
ATP 0.4 mM	0.3 ± 0.03	13.3 ± 0.98	10
UTP 0.4 mM	$2.4 \pm 0.6^{**}$	$9.0 \pm 0.45^{**}$	5
ITP 0.4 mM	$19.3 \pm 2.4^{***}$	$3.5 \pm 0.17^{***}$	5

Asterisks denote the degree of significance for the differences between the means obtained with ATP and those obtained with UTP and ITP.

$^{***} = P < 0.001$, $^{**} = 0.001 < P < 0.01$, $^{*} = 0.01 < P < 0.05$.

7. *The effects of ITP and UTP in the presence and absence of zinc.*

It is possible to induce contraction in actomyosin systems with other nucleoside triphosphates than ATP, viz. acetyl-ATP, CTP, GTP, ITP and UTP (BERGKVIST and DEUTSCH 1954, PORTZEHL 1954, RANNEY 1955, HASSELBACH 1956). In the following experiments the contraction-inducing effects of ATP, ITP and UTP on glycerol-extracted fibre bundles are compared under strictly isometric conditions. Furthermore, it is shown that relaxation may be obtained with zinc in the presence of UTP and ITP instead of ATP.

Table III shows the results obtained with 0.4 mM UTP and ITP as contraction-inducing substances, including for comparison the corresponding results with 0.4 mM ATP. A practically constant level was reached 3 min after the start of the contraction, both with ATP, UTP and ITP. In accordance with results of HASSELBACH (1956), ITP gives the weakest isometric contraction and ATP the strongest, while UTP lies between. It is remarkable that the latency, *i. e.* the time from the application of the nucleotide until the start of contraction is considerably longer with UTP and, in particular, ITP than with ATP.

Application of zinc in a final concentration of 0.2 mM in the presence of 0.4 mM UTP induces total relaxation of a fibre bundle

that has been previously contracted with 0.4 mM UTP. The relaxation is complete within the course of one minute (without pretreatment with zinc). After 30 min washing with zinc-free buffer solution, a new contraction can be induced with 0.4 mM UTP.

Zinc in 0.2 mM concentration in the presence of 0.4 mM ITP also induces relaxation of a fibre bundle previously contracted with 0.4 mM ITP or 0.4 mM ATP, in the latter case followed by 15 min washing with ATP-free buffer solution. The tension returns to the initial level within only about 15—30 sec (without pretreatment with zinc). For the rate of relaxation after treatment with zinc, see later (Results, 8). A new contraction can be induced with 0.4 mM ITP or ATP after 30 min washing with zinc-free buffer solution.

Thus, under the conditions used here, isometric relaxation can be induced with zinc in the presence of UTP and ITP instead of ATP. This is in contrast to the findings obtained at relaxation with the Marsh-Bendall factor and EDTA. According to HASSELBACH (1956), who used a semi-isometric recording technique, ITP and UTP in concentrations up to 10 mM are unable to induce relaxation of contracted fibres in the presence of Marsh-Bendall factor and 10 mM magnesium. WATANABE and SLEATOR (1957) report that the combination 4 mM EDTA + 4 mM magnesium + 4.8 or 8.1 mM ITP stops the isotonic contraction of a glycerol-extracted fibre bundle induced by 8.1 mM ITP + 4 mM magnesium, but does not produce significant relaxation of the bundle as does 4 mM EDTA + 4 mM magnesium + 4 mM ATP. Results similar to those of WATANABE and SLEATOR were obtained by BENDALL (1958 b).

8. *Visco-elastic effects induced by zinc and ITP.*

The visco-elastic effects of zinc + ATP have been described earlier (EDMAN 1959). Because of the pronounced relaxation obtained with ITP it is of interest also to investigate the effect of zinc + ITP on the visco-elastic behaviour at and following quick stretch. For the performance of the experiments, see Methods III. Table IV summarizes the results of the visco-elasticity measurements performed with 13 fibre bundles. For comparison the results published earlier (EDMAN 1959) with 0.2 mM zinc + 0.4 mM ATP have also been included in the table. In the interpretation of the

Table IV.

Effects of zinc + ITP and zinc + ATP on the visco-elastic behaviour of resting glycerol-extracted muscle fibre bundles subjected to stretch.

Test medium	1	2	3	4	5	6	7
	Equilibrium tension of fibre bundle *before* stretch g/cm²	Stretch in % of initial fibre length	Initial tension increase at stretch g/cm²/% stretch	Equilibrium tension increase after stretch g/cm²/% stretch	Half time for tension decrease in sec	Number of expts.	Mean area of cross section cm². 10^5.
Veronal buffer alone (1 mM Mg)	108±3	0.83	506±39	259±36	4.1±0.3	13	20.7 ± 0.52
Zn 0.2 mM + Mg 1 mM + ITP 0.4 mM	86±5***	0.83	299±9***△△△	128±4***△△△	1.3±0.1 ***△△△	13	19.6 ± 0.36
Zn 0.2 mM + Mg 1 mM + ATP 0.4 mM	73±7***	0.83	642±33*	188±12	6.3±1.0*	13	20.0 ± 0.91

The degree of significance has been calculated for the differences between the means. Asterisks denote differences between tests (Zinc + ITP or zinc + ATP) and controls (veronal buffer alone); triangles denote differences between the tests.

$***△△△ = P < 0.001$, $* △ = 0.01 < P < 0.05$.

Table V.

Visco-elastic parameters for the model used (see text) calculated from data of Table IV.

Test medium	C_1	C_2	ϱ
Veronal buffer alone (1 mM Mg)	1	1	1
Zn 0.2 mM + Mg 1 mM + ITP 0.4 mM	1.7	2.4	6.3
Zn 0.2 mM + Mg 1 mM + ATP 0.4 mM ...	0.79	2.0	0.74

results the same model was used as earlier (EDMAN 1959) in the analysis of the visco-elastic conditions, *i. e.* an elastic element of compliance C_1 in series with an elastic element of compliance C_2, that acts in parallel with a viscous element of fluidity ϱ and with a force F, which represents the active contractility. Table V shows the effect of the treatment on C_1, C_2 and ϱ computed from the mean values given in Table IV. As pointed out earlier (EDMAN 1959), no significant visco-elastic effect is obtained with 0.2 mM zinc + 0.4 mM ATP. The combination of 0.2 mM zinc + 0.4 mM ITP, on the other hand, gives increased compliance in both C_1 and C_2 and also increased fluidity (ϱ) of the viscous element.

It was demonstrated earlier (EDMAN 1959) that visco-elastic factors are not rate-limiting in the isometric relaxation process with 0.2 mM zinc + 0.4 mM ATP, and it was considered probable that the *isometric* relaxation of a glycerol-extracted fibre bundle induced by zinc + ATP is mainly the disappearance of a force probably not dependent upon visco-elastic factors. In accordance, the decrease in tension obtained with 0.2 mM zinc + 0.4 mM ITP is not more pronounced than that produced by 0.4 mM zinc + 0.4 mM ATP (see column 1, Table IV), although the visco-elastic effects are significantly greater with the zinc + ITP combination.

Fig. 10 illustrates the rate of relaxation after contraction and the rate of tension decrease after stretch with 0.2 mM zinc + 0.4 mM ITP and with 0.2 mM zinc + 0.4 mM ATP. (The stretch experiments with zinc + ITP are the same as those presented in Table IV; the stretch and relaxation experiments with zinc + ATP are the same as those published in Fig. 6, EDMAN 1959.) As is seen, the relaxation takes place at practically the same rate with the combinations zinc + ATP and zinc + ITP studied notwithstanding that the latter gives an appreciably more rapid

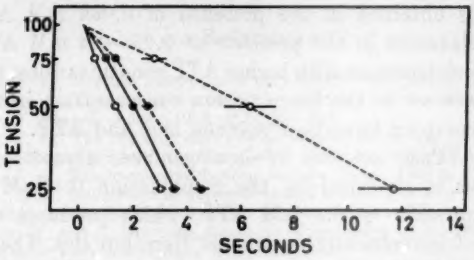

Fig. 10. Comparison between the decay of tension after stretch of resting glycerol-extracted muscle fibre bundles and the relaxation of isometrically contracted fibre bundles in the presence of 0.2 mM zinc + 0.4 mM ITP and of 0.2 mM zinc + 0.4 mM ATP.

Abscissa: time in sec after start of the relaxation and start of the tension decrease after stretch, respectively.

Ordinate: tension remaining in excess of the equilibrium tension, expressed in percentage of total tension decrease.

Number of experiments in respective groups: 13. The standard error of the mean is indicated with a horizontal bar.

o——o tension decrease after stretch, zinc + ITP
•——• relaxation after contraction, zinc + ITP
•- - - - - • relaxation after contraction, zinc + ATP
o- - - - - o tension decrease after stretch, zinc + ATP

decrease in tension after stretch. Since the fibre bundles were pretreated with zinc before the relaxation was started (see Methods III), limitation of the relaxation rate caused by diffusion of zinc into the bundles was avoided. However, it cannot be excluded that the rate of relaxation is limited instead by the diffusion of ITP and ATP into such zinc-loaded fibre bundles. Therefore, it is not possible to decide from these results whether the rate of relaxation for the individual contractile unit differs with 0.2 mM zinc + 0.4 mM ITP and 0.2 mM zinc + 0.4 mM ATP.

Summary.

1. The relaxing effect of 0.1 mM and 0.4 mM zinc on previously contracted, glycerol-extracted rabbit psoas fibre bundles was studied under isometric conditions in the presence of 1 mM magnesium and varied concentrations of ATP (0.0031—6.4 mM). No relaxation is obtained in the absence of ATP. A certain, partial

relaxation is obtained in the presence of 0.006 mM ATP and complete relaxation in the presence of 0.05—0.4 mM ATP. The relaxing effect decreases with higher ATP concentrations, probably due to a decrease in the free zinc ion concentration in the bath caused by complex formation between zinc and ATP.

2. The ATPase activity of homogenized, glycerol-extracted rabbit psoas is abolished by the combination 0.1 mM zinc + 1 mM magnesium + 0.2 mM ATP, which produces complete relaxation of isometrically contracted fibre bundles. The ATPase activity is retained with the combination 0.1 mM zinc + 1 mM magnesium + 2.6 mM ATP, which gives no relaxation.

3. Zinc and magnesium show mutually antagonistic action upon the contractility in glycerol-extracted fibre bundles. The relaxing effect of zinc is probably not due to a simple competition with magnesium.

4. The lowest zinc concentration found to give complete relaxation of a contracted fibre bundle is 0.0125 mM if magnesium is absent and 0.04—0.08 mM ATP is present.

5. Calcium in 10 mM concentration increases the rate of the relaxation induced by 0.2 mM zinc + 1 mM magnesium + 0.4 mM ATP. No significant influence of 1 mM and 0.1 mM calcium was demonstrated.

6. Inorganic pyrophosphate (PP) and tripolyphosphate (TPP) in concentrations of 0.25 mM and 1 mM induce relaxation of contracted fibre bundles. Adenosinediphosphate (ADP) in concentrations of 0.25 mM, 1 mM and 10 mM does not give any significant relaxation in excess of the drop in tension resulting from mere removal of ATP by washing. The presence of 0.2 mM zinc does not modify the action of ADP, PP and TPP.

7. Relaxation can be induced by 0.2 mM zinc in the presence of 1 mM magnesium and 0.4 mM inosinetriphosphate (ITP) or uridinetriphosphate (UTP) instead of ATP.

8. The effect of 0.2 mM zinc + 1 mM magnesium + 0.4 mM ITP on the visco-elastic behaviour of resting fibre bundles subjected to stretch was studied. There is an increase of the elastic compliance and also an increase in fluidity of the viscous resistance of the fibre bundle.

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On the Physiology of the Cutaneous Sensory Modalities.

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Abstract.

BING, H. I. On the physiology of the cutaneous sensory modalities. *Acta physiol. Scand.* 1959. *46*. 88—96. — Some effects of direct cutaneous application of adrenaline, noradrenaline, acetylcholine, atropine and histamine on the sensory modalities, especially cold spots and pain are reported. After application of adrenaline or noradrenaline the number of cold spots increases. A crossed reflex occurs on the opposite side of the body with exactly the same reactions. Trilafon nullifies the effect on cold spots both at the original site and at the symmetrical contralateral site. The increase disappears slowly in the course of 3—4 days. It is noticeably longest on the reflexly affected side. Also acetylcholine increases the number of cold spots both at the original site and at the symmetrical contralateral site. This increase disappears extremely rapidly. Application of atropine reduces considerably the number of cold spots. Here is also a symmetrical reflex. By applying histamine to the skin the cold spots are reduced or vanish on both sides. At the same time there arises a characteristic change in the sensitivity of the skin with superficial hypaesthesia, deeper hyperaesthesia and hyperalgesia. It is shown that histamine is released in the area in question and that the reflex itself can be explained in this way.

The physiological concept of the skin's sensory modalities was, until recently, fairly certain. After BLIX, GOLDSCHEIDER and VON FREY had established that the four modalities — warm, cold, touch and pain — were released from different "spots" in the skin, anatomical investigations revealed heterogeneous nerve endings which were considered to be the receptors for the various cutaneous sensations. Even in recent reference books these opinions are accepted, and pictures of these various end-organs are shown. Electrophysiological research has supported this view.

However, from various different quarters in recent years, fresh anatomical research has shown that the earlier suggestions do not hold good. In particular SINCLAIR (1955) came to the conclusion that sensory impressions are transmitted from a finely divided, undifferentiated network of nerves in the skin. A new explanation must then be found for the distribution of the sensory modality spots in the skin.

I. *Investigations of cold spots in the skin.*

The investigations presented here are a continuation of the research of BING and SKOUBY (1950) and BING (1954—57). The first study demonstrated that the number of cold spots varied with the temperature of the skin. In the next, it was shown that such a change was induced by the intracutaneous application of substances with autonomic effects. The last paper reported that a suitable solution of histamine chloride applied to the skin resulted in an appreciable decrease in the number of cold spots. Mechanical friction and ultra-violet treatment have the same effect because they release histamine in the skin. Finally, a symmetrical reaction was demonstrated in that the effect on the corresponding place on the other side of the body was identical in every way with that on the area to which histamine was applied.

On the basis of these experiments it seemed of interest to investigate the effect of direct application to the skin of substances known to affect the number of cold spots after intracutaneous application.

Method.

By means of a rubber stamp a 4 cm area was divided into squares of 1 cm usually on the volar surface of the lower arm. The testing device consisted of a brass container with a conical point, the end of which

had a flat surface measuring 1 mm². In the container was water at a temperature of about 10° C.

After the skin has been exposed for ten minutes, a solution of the substance to be examined is applied to the skin, in that a drop is placed within the marked area and distributed within this limit. The liquid should be allowed to evaporate before ascertaining the number of cold spots. The effect is immediate, and it is also possible to observe immediately a changing sensitivity on the reflexive places (considerable increase or decrease of cold spots). Furthermore, the area can be delimited from the surrounding skin and the number of cold spots counted.

Results.

Direct application of adrenaline. After counting the cold spots on the experimental area, a solution of adrenaline 1 : 1,000 was applied. A considerable increase in the number of cold spots usually occurred. In 13 experiments there was an average of 25 cold spots before and 42 after the application of adrenaline. On single occasions no increase took place. There was a similar increase in the number of cold spots in the symmetrical contralateral untreated skin area. In 10 cases there was an average of 15 cold spots prior to and 30 after the application of adrenaline to the opposite limb. Cold applied to the affected skin areas was felt colder than when applied to surrounding areas. By searching outside the reflexly affected area with the test container, one or two small areas were usually found which felt cold and which had a large number of cold spots. Furthermore, similar symmetrical areas were found on the side treated with adrenaline which also felt colder and had an increased amount of cold spots. On both sides the increase in cold spots diminished slowly, being demonstrable for several days. The increase was maintained considerably longer on the reflexly affected than on the directly treated side.

In four experiments, the effects of the various applications of adrenaline lasted as follows:

The treated cutaneous area	The symmetrical contralateral cutaneous area
48 hours	96 hours
48 »	96 »
24 »	72 »
48 »	96 »

Direct application of Trilafon (Schering), Sch. 3940 (Parphazine).

Trilafon is a derivative of chlorophenothiazine. It is used as a substitute for chloropromazine, and has an anti-adrenaline effect when injected intravenously, and it was therefore decided to test the substance on the skin in the form of a 2 per cent ointment. The cold spots were counted before and after application. All the experiments gave similar results. In 14 tests an average of 20 cold spots was found. After applying Trilafon there were no cold spots. Similarly in seven experiments in which the cold spots were counted and Trilafon then applied to the opposite side, a reduction of cold spots was observed. In cases where the cold spots were increased by an application of adrenaline, they vanished after an application of Trilafon.

It could be surmised that Trilafon had an atropine-like effect on acetylcholine. This was not the case, as was shown by applying acetyl-beta-methylcholine and then Trilafon. In 8 experiments, the first count yielded an average of 20 cold spots. After methylcholine 31, and after a subsequent application of Trilafon an average of 33 cold spots were counted. This result shows that there was no effect on the acetylcholine.

Direct application of noradrenaline.

A solution of 1 : 1,000 was used with the same results as in the case of adrenaline. In six experiments 19 cold spots were found before and 41 after the application of noradrenaline. In seven tests there was an average of 29 cold spots before and 52 after application of noradrenaline to the symmetrical contralateral site.

Experiments carried out over some days showed that with noradrenaline as well, the number of cold spots diminished slowly and that they were maintained in the highest numbers on the site which did not receive the application. An increased number of neighbouring reflex spots was also found on both sides of the body, and at these sites the cutaneous sensibility and muscle tonus were reduced.

Direct application of an acetylcholine preparation.

Acetyl-beta-methylcholine was used, being more resistant to cholinesterase, in a 5 per cent solution. There was a notable increase in the number of cold spots. In seven experiments the

number prior to the application was an average of 18 and afterwards of 43 cold spots. It was then apparent that this increase diminished very quickly. After five minutes the number was down again to 18. No explanation can be offered as to why methylcholine's effect on the cold spots vanished so quickly in contrast to the effect on sensibility and muscle tone.

A similar symmetrical reflex action occurred on the opposite arm and there too the increase in the number of cold spots diminished rapidly. In three experiments an average of 16 cold spots was located, which increased to 33 after application of methylcholine to the opposite side. After 5 min. the total had shrunk to 14. In some cases a solution of 1 : 1,000 Synstigmine (Prostigmine) was applied prior to the methylcholine. The effect then lasted for some hours and vanished slowly.

Direct application of 2 per cent atropine sulphate.

After application of this solution a considerable decrease in the number of cold spots was observed, the number in most instances falling to zero. When, after about 1—2 hours, the atropine was reabsorbed, the earlier number of cold spots could be found. Seven experiments yielded an average of 19 cold spots prior to treatment with atropine, which afterwards were reduced to an average of 2.

Investigations on the symmetrical contralateral site gave the result that here there was a reflex action with a decrease in the number of cold spots. Eight experiments carried out in the usual way gave an average of 19 cold spots prior to and 6 after an application to the opposite arm. On a number of occasions, it was observed that after a second application of atropine as soon as the effects of the first had diminished, there followed no reduction in the number of cold spots. In cases where adrenaline was used to increase the cold spots, these were affected by atropine in the same way. The decrease here was just as marked as that described above.

II. *Investigations of pain spots in the skin.*

Previous investigations (Skouby 1951) concerned the threshold of the pain modality as influenced by increased temperature. The action of acetylcholine preparations was demonstrated. It

reduced the pain threshold when administered intracutaneously or by iontophoresis. The supposition was advanced that changes in the concentration of acetylcholine normally released in the skin might affect the susceptibility to pain. The subject of SKOUBY's second paper (SKOUBY 1953) dealt especially with the effects of acetylcholine and histamine when injected simultaneously. It was shown that pain was produced in this way.

GOLDSCHEIDER has described in a series of papers (1915, 1916, 1917) how a characteristic reflex can be released by pinching a fold of the skin with the fingers or with a clip. He made many experiments covering the whole body surface, partly on himself and partly on others who were particularly suitable and points out that a spinal reflex occurs which shows the same segmental distribution as others have found. By treating an area of the skin with the finger or clip as mentioned above, pain is induced which gradually disappears. Subsequent changes in sensitivity occur both proximally and distally to the treated area. GOLDSCHEIDER found at the places where the reflexes occurred the following: — To slight pressure there is a hypaesthesia. With somewhat harder pressure a hyperaesthesia and with firm pressure a hyperalgesia. Thus, they are phenomena identical to those produced by the application of histamine.

Next must be mentioned how the sensitivity of the skin to pain can be altered by influences on the skin.

Results.

A uniform sensation is usually experienced by letting a finger pass lightly over the skin. Upon firmer pressure, with the nail or a pointed object, slight pain is felt which quickly disappears. Firm pressure is then applied with the pulpa of a finger to a place on the skin for about 30 sec. A change in the sensitivity of the skin at this spot can then be distinctly felt, and by rubbing it gently a hypaesthesia is found. With somewhat firmer pressure a hyperaesthesia and under hard pressure a hyperalgesia is observed, which slowly diminish.

Exactly the same changes in sensitivity occur when a solution of histamine (1 : 1,000) is applied to the skin. However, with a previous application of antihistamine these phenomena do not occur either by these methods or by mechanical influences.

These investigations are easy to carry out and give pronounced results. The results are based on experiments involving many individuals.

The changes in sensitivity mentioned here occur also as a symmetrical reflex on the opposite side of the body. (This has also been shown in an earlier paper dealing with the cutaneo-muscular reflex.) The release of histamine, which occurs so easily through mechanical influences, must be assumed to be of physiological significance.

In the experiments we have carried out on reflex changes we have found the same results as GOLDSCHEIDER (1915, 1916); we have, however, not used clips. The same result can be achieved by applying pressure to the skin with the fingers, and letting the pressure remain until the pain decreases.

Then the following experiment was made:

An area is marked on the volar side of the arm where pressure will be applied, and 5 cm below this two more areas are marked. On these last two places an antihistamine (paradryl) solution is applied. Next, strong, steady pressure which induces pain is applied to the first area. After a few minutes the pain diminishes. One can then recognize proximally and distally, the zones and phenomena described by GOLDSCHEIDER (1915, 1916). However, in these zones there are two islands, where the phenomena cannot be shown, these are where paradryl has been applied. Histamine cannot reach the receptors as these are blocked by antihistamine.

GOLDSCHEIDER did not observe what we have been able to demonstrate that there is a crossed reflex at the symmetrical contralateral site, covering the same area and showing the same triad. Here too, can be found the two islands without these changes in sensitivity. Antihistamine on the other arm has also prevented the corresponding places on the reflexly affected site from producing histamine.

GOLDSCHEIDER's detailed and ingenious method of experimenting has in this case, as so often before in his research, yielded rich results. Inspired by this, some investigations of reflexes published previously by the author have been taken up.

In this research (BING 1935), an area is found on the abdomen where a hyperalgesia can be induced. This zone corresponds to the colon. Mechanical influences upon the zone result in a change in the tonus of the underlying colon. Also on the thorax zone a hyperalgesia is found, corresponding to the underlying lung (BING

1936). Here also, a cutaneo-pulmonary reflex can be induced. In these cases it is probable that histamine plays a role. This has been substantiated by the fact that apart from a hyperalgesia in the areas in question, there also is found a hypaesthesia upon slight pressure and a hyperaesthesia. It therefore must be presumed that a reflex from the viscera to the areas in question has released histamine.

Discussion.

As mentioned in the introduction, investigations in recent years have disproved the theory that special receptors, "sense bodies", exist from which the various modalities are transmitted further by specific nerve fibres. On the other hand, it has been proved that there is a dense undifferentiated network of nerve fibres in the outer layer of the skin. It is in these that the receptors for the various modalities must be presumed to occur.

In this paper it is shown how the skin's sensitivity can be affected by the application of various substances. Most of these substances are present in varying amounts in the skin. It is shown that in some cases these substances can be supplemented so that there is an increased reaction. In other instances the substances have a reducing effect in relation to the influence of the substances which are already present in the skin, such as for example that effect of histamine which prevents the cold-reacting substance (adrenaline) from acting. It is therefore presumed that these substances might by direct application cause a specific influence on the peripheral nerve endings and that this influence is lead specifically further.

This hypothesis is substantiated by the observations made concerning the crossed reflex. It is seen how the same substances are activated reflexly at the symmetrical contralateral site as well as at the site treated with the solution.

Some experiments were done at the Neurophysiological Institute and at the medical department of the Finsen Institute in Copenhagen.

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